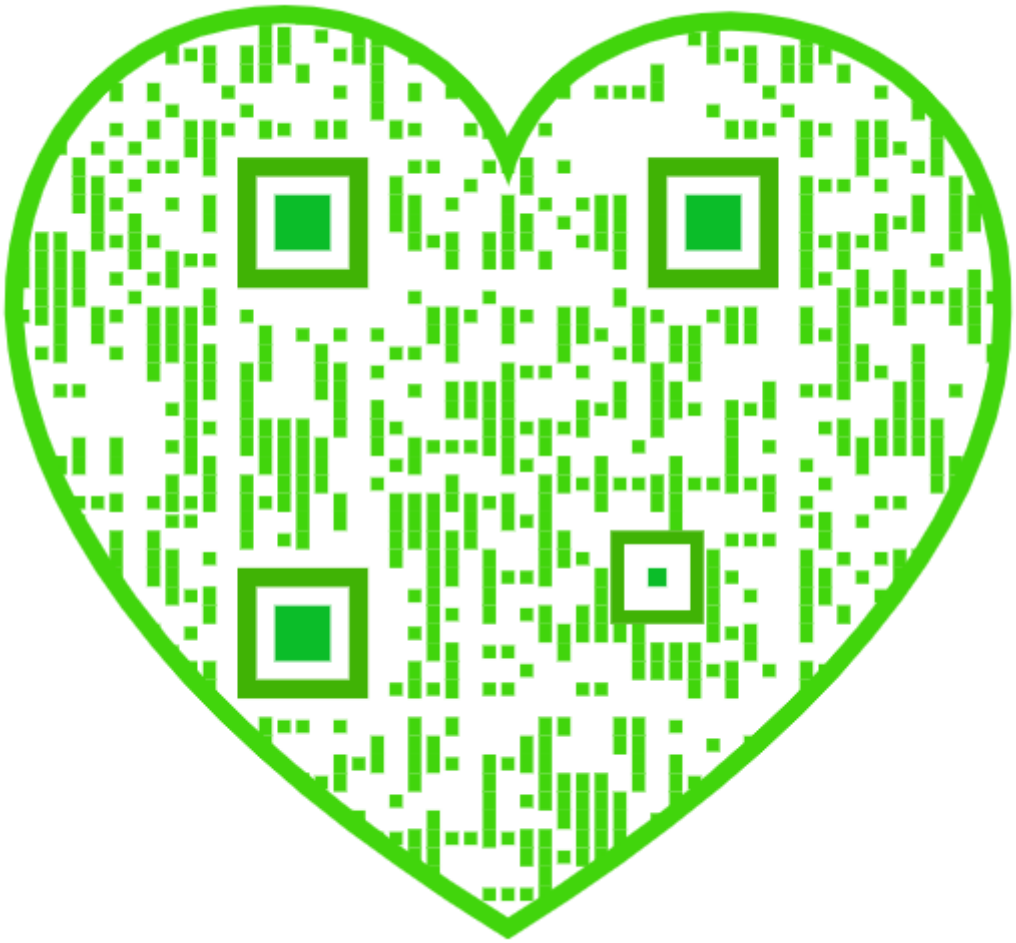

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BIOLOGICAL STAINS

A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory

By H. J. CONN

Secretary, Biological Stain Commission

Prepared with the collaboration of
Various Members of the
Biological Stain Commission

SIXTH EDITION

Revised with the assistance of

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PREFACE

The general purpose of this book was well explained in the preface to its first edition, and that preface is, therefore, reprinted on the following pages. The various editions which have followed have seen a gradual enlargement of the book, with greater accuracy of detail; but its purpose and general scope have not been changed.

A longer period (seven years) has elapsed since the fifth edition than occurred between any other successive two. This has not been so much because of the lack of new material on the subject as because of difficulty in organizing that material in the best form for presentation. There is a smaller increase than usual in the number of new dyes described in this edition; but this is offset by the addition of histochemical reagents which are not dyes in themselves, but develop color when properly applied to tissue containing the compounds which the reagents are intended to demonstrate.

Although reference is given to certain histochemical reagents, the general subject of histochemistry (in spite of its growing importance) is treated very sketchily. This book hardly seems the place to consider that field in any detail, especially considering that new text books dealing with the subject alone and written by specialists in the field, are now beginning to appear. Nevertheless, one of the important changes in this edition, in comparison with the fifth, is in its references to histochemical usage of certain dyes and dye-like reagents.

Perhaps the greatest change in this edition is the greater stress laid on methods of identification of stains. All previous editions have referred to spectrophotometric measurements and their use in characterizing dyes; but beyond giving the absorption maxima (sometimes not fully verified) under the individual description of the stains, such data were largely omitted. At present, the Biological Stain Commission has much more material on this subject available, from data that it has been collecting during the last five years in its laboratory at the University of Rochester Medical School, and it seems worth while to include some of that material in this book. As a result, this book contains some 20 illustrations in which spectral graphs of nearly all the stains now on the certification basis are included. In every case these graphs are of typical samples of the stain in question, selected after a comparison of numerous batches of the same dye which have been accumulating in the Commission laboratories for twenty years or more. These curves are very useful to the Stain Commission in the identification of stain samples submitted for certification; and it is hoped that their inclusion here may increase the familiarity of users with the optical properties of dyes and help them to use the stains more intelligently.

Chromatography, a still more recent method of dye identification, is discussed in this edition for the first time. It is not treated extensively, however; its importance is appreciated, but methods of applying it have not yet been sufficiently standardized to justify its routine use in stain identification. Perhaps the brief references to the method given in this edition will stimulate the research the method needs to make it a more useful tool in this work.

Users of the book will also notice that there has been a slight rearrangement of the material, with the chapter on Theory of Staining placed earlier in the book and one additional chapter added to include Miscellaneous Dyes, Pigments and Histochemical Reagents.

H. J. Conn

Geneva, N. Y.
October, 1952

PREFACE TO FIRST EDITION

When microscopists first began, in the sixties and seventies, to use stains, the demand for dyes for this purpose was naturally too small to justify a special source of supply. They therefore had to make use of textile dyes, which were then very crude and were not constant in their composition. After a number of years, however, the demand for biological stains grew and a special commercial source of supply for them first appeared in Germany. This was the Dr. G. Grübler and Co. This company did not manufacture the dyes, as used commonly to be thought in other countries; but on the other hand it cannot be denied that its founder made a distinct contribution to science in making the first effort to secure constancy and reliability in dyes intended solely for the use of the microscopist. It is supposed that he tested dyes under the microscope himself, and if a batch proved satisfactory in his experience bought a supply large enough for a number of years, bottled it under his own label and sold it to biologists. There is no question but that in this way the biologist was furnished with a much more reliable line of stains than if he had been obliged to buy directly from the dye manufacturers; but it was an empirical method of standardization and there was nothing to prevent different batches of some dye secured by this company from varying considerably in their composition. Such upon investigation has proved to be the case.

Although a great service was done to biologists by this company in the latter part of the nineteenth century, such methods of standardization are not in keeping with modern scientific knowledge. A recent coöperative undertaking has therefore been organized in America to put the standardization of stains upon a scientific basis. This undertaking started after the war had caused a shortage of stains, with the object of securing a reliable supply when the foreign sources were unavailable. It has since then been widened in its scope; and now that the foreign products are again available, the purpose of the work is to effect a scientific standardization of stains whether derived from foreign or domestic sources. As a matter of fact, so far only domestic samples have been considered. This has not been because of any prejudice against foreign stains, but because of practical difficulties; it is, in brief, difficult to test each batch before it is put on the market when the concern handling it is in Europe.

The organization through which this work is being carried on is known as the Commission on Standardization of Biological Stains. It was organized in 1922 under the auspices of the National Research Council and is still affiliated with it, although now no longer a part of the larger body. It is in effect a coördinating com-

mittee representing the American Chemical Society, the American Society of Bacteriologists, the Society of American Zoologists, the Botanical Society of America, the American Association of Pathologists and Bacteriologists and the American Association of Anatomists. It has a membership of about sixty biologists, members of the various societies just mentioned, who assist in the examination and testing of stains, each in those particular lines of technic with which he is especially familiar. It has secured the coöperation of chemists, dye manufacturers and stain dealers, so as to be sure that the needs of biologists can be immediately reflected in the supply of stains on the market. Its affairs are managed by an executive committee of five members, the present members of which represent bacteriology, botany, dye chemistry, pathology and zoology, respectively. This executive committee has undertaken the preparation of this book. The authorship of the book has been assumed by the chairman of the committee, however, in order to fix the responsibility and to make bibliographic references to it simpler than in the case of plural authorship; but the assistance of the other committee members in the work has been so great that they may be practically considered coauthors of the book. The chairman of the committee, therefore, wishes to take this occasion to acknowledge the invaluable assistance given by these other members. Without their coöperation such an undertaking would have been impossible.

The chief object of the book is to present in logical form the information which has been accumulating in the hands of the Commission since it was organized. It is neither a treatise on dye chemistry nor one on microscopy; although it contains information in both fields. It is an effort to present in a form acceptable to biologists the principles of dye chemistry so far as they have a bearing on biological stains; and to discuss the suitability of the different dyes for various biological purposes, presenting data partly original and partly drawn from the literature. The subject matter is realized to be incomplete, particularly that part of it which deals with the biological uses of dyes. An effort has been made to list the most important present uses of stains, and of the obsolete uses to mention those of historical significance; but it is realized that there must be many omissions. It is hoped that readers of the book will coöperate by calling to the author's attention places where the treatment of any subject seems inadequate.

H. J. Conn, *Chairman,*
Commission on Standardization
of Biological Stains.

Geneva, N. Y., 1925

CHAPTER I

USES AND STANDARDIZATION OF BIOLOGICAL STAINS

WHAT ARE STAINS?

A QUESTION frequently asked is, "what are biological stains and how do they differ from dyes?" The answer is simply that they *are* dyes, but are adapted for very special purposes. The manufacturer thinks of two classes of dyes, those intended for general purposes and those specially adapted for biological use. To him the "biologicals" are a class in themselves, notable chiefly because of the care with which they must be manufactured and the necessity of having them agree with rigid specifications so that they will be satisfactory for the delicate procedures in which they are employed. To the biologist, however, there are several classes of products included among the "biologicals" and not all of them are stains; there are medicinal dyes, bacteriostatic agents, and indicators, which are not truly stains but are put in the same group by the manufacturer. Strictly speaking, however, a biological stain is a dye used for making microscopic objects more clearly visible than they would be unstained. Such a dye may also serve for textile purposes as well; but usually a special grade is needed for biological use. The same statement is equally true (perhaps more so) of medicinals and indicators; and since chemically they are all very similar they are all considered in this book. Chief stress, however, is laid on those which are employed primarily as stains.

THE HISTORY OF STAINING

Considering how dependent microscopists are today upon the use of stains, it is hard to realize that much important work had been done with the microscope before the use of stains was attempted. Although natural dyes such as carmine and indigo were well known in the early days of the microscope, their use in staining microscopic preparations does not seem to have become common till about 1850; and anilin dyes were not put on the market until 1856. Yet anyone who has studied the history of biology must realize that many discoveries had been made with the microscope before this period.

It is safe to say, nevertheless, that the use of stains revolutionized microscopic technic. The early microscopists were able to make much progress without stains because of their painstaking diligence. The work without stains must have been extremely difficult, and it is hard on reading some of the old publications to believe that some of the minute structures described were actually seen. Few users of the microscope today would be likely to have either the patience or the eyesight to do the work described in

those early days. The fact that the microscope is now being used successfully in the hands of so many students who would not think of comparing themselves with the pioneers in microscopy is due to the use of stains more than to any other factor—although, of course, no one can deny that modern improvements in the microscope have also played a part of great importance.

As a matter of fact, a revolution of a different sort, which has no relation to staining, is in progress at the present time. The last few years have brought out more radical changes in microscope design—including that of the electron microscope—than have been seen for the greater part of a century. What will be the outcome of such radical departures as the use of electron beams, X-rays, phase differences, reflecting optics, is still impossible to predict. One might almost assume that the use of improved types of microscopes would eventually eliminate the need of stains to render objects more easily visible—were it not for the equally rapid development in histochemistry, in which field dyes or similar reagents are apt to be needed for a long time to come.

The present is hardly the place to go into the history of staining in any detail; it has been adequately treated elsewhere. Gierke (1884) was the first to discuss the subject, and presented a fairly detailed account of the early uses of biological stains, as they were understood at that time in Germany, and brought the subject up to the date on which he wrote. Conn (1933, 1948) has presented a rather more cursory review on staining history, up to modern times, based on a study of moderately recent publications without making any search through old texts. Such a search has been made recently, however, by Lewis (1942) who has presented the most thorough review of the beginnings of staining that has yet appeared, and shows that its origin can be traced way back to Leeuwenhoek, the "father of the microscope", and the first scientist to see bacteria.

Histological technic, as we know it today, seems to have become fairly well understood about 1860, and it embraced the use of certain natural dyes, notably carmine, but also indigo, madder, saffron and phytolacca. This rapid development of the subject followed a paper by Gerlach (1858) in which he particularly called attention to importance of staining (with carmine) in histology. It is not certain whether or not Gerlach thought he had originated the idea; but it is clear that he was hailed as the father of staining by many of his contemporaries, particularly in Germany. Careful students of the subject, however, like Gierke, took pains to explain that though Gerlach had established (eingefürt) staining technic, the actual use of dyes in microscopic work preceded him. Gierke cites Göppert and Cohn (1849) as the earliest users of biological stains. Subsequently, however, the introduction of stains into microscopic work has been ascribed to Ehrenberg (1838);

then to Hill (1770); while now Lewis (l.c.) quotes from a letter written by Leeuwenhoek to the Royal Society in 1714 (published 1719) in which he mentions staining sections of muscle fibers in saffron to increase the visibility of the particles as viewed under one of his high power lenses.

One can never be certain how long any particular individual will remain credited with being the "first" to do something; but it is difficult to see how the use of dyes in microscopy can precede the invention of Leeuwenhoek's microscopes.

Following the work of the pioneers in staining the development of the subject was rapid, particularly after hematoxylin had been introduced by Waldeyer (1863) and more successfully by Böhmer (1865), anilin dyes by Beneke (1862) and alcohol differentiation by Böttcher (1869).

Gierke (1884), in his historical discussion of staining, says that the history up to his day was divided into three periods, each occupying a decade. The first decade, the fifties, was characterized by a few important but unrelated discoveries which ended in the work of Gerlach—each investigator following up accidental observations on the staining powers of carmine and the other well known dyes of those days. After Gerlach's work, the development of the technic in the sixties was more rapid and depended less upon chance success by the individual investigator; the effort was made to use similarly all the dyes and metallic colors then available. The next decade would have had much less left to develop in this line if it had not been that by this time the great variety of anilin dyes were available and microscopists were constantly finding new uses for them. Gierke wondered if there would be any opportunity for equal development during the ten years to follow his paper.

That development did not stop in his day is well known. Scarcely a year has passed without the introduction of some new staining technic of considerable importance. Sometimes dyes hitherto unknown to the biologist have been shown to be valuable in bringing out some particular structure; at other times new combinations of dyes have proved of special value for other purposes; while by other investigators it has been shown that old methods used with modern refinements of apparatus and technic, may bring out details not dreamed of by the early histologists.

USES OF STAINS

Only a brief summary of the uses of stains is given here. More detailed citations are given under the discussion of the individual dyes throughout this book. Certain very general texts of the subject are also useful; among those which have proved most valuable sources of the information given in these pages are: Mann (1902),

Ehrlich (1910), Krause (1926–7), Lee (1950, and earlier editions), McClung (1951, and earlier editions).

Animal microtechnic. Although the earliest uses of stains were botanical, modern histological technic was first developed on zoological material. As a result the first extensive use of stains was in animal histology. It is still true that there are many more staining procedures developed in animal histology than in plant histology, and that many more dyes are thus employed in the former field than in the latter. (This is not true, as will be explained below, in regard to cytological methods.)

In animal histology one thinks first of the *general tissue stains.* These involve the use of one, two, and occasionally three dyes in staining sections of general animal tissue designed primarily to differentiate nuclei from cytoplasm of cells and to permit distinctions between the various types of tissue. Best known of these are the various procedures calling for hematoxylin, sometimes alone and sometimes with a counterstain, such as eosin Y, Congo red, or safranin; the hematoxylin-eosin combination is in such general use that it is commonly referred to as the "H-E stain". Although these hematoxylin procedures have received many modern refinements, they date back to the 19th century and still bear the names of Heidenhain, Delafield, and Mayer, men who did their chief work in the eighties and nineties. Also included among the general tissue stains are various combinations of basic dyes such as crystal violet, methylene blue or one of the azures, with some contrasting acid dye such as eosin Y; the former to stain the nuclei, the latter, the cytoplasm of the cells.

Somewhat more specialized in their application are the *connective tissue stains.* The distinction between them and the general tissue stains is more or less arbitrary, particularly because some procedures that bring out connective tissue and elastin to good advantage are also fine general tissue stains. The grouping can, however, be made in a rough way and for practical purposes is rather convenient. Of special note among the connective tissue stains is a triple-staining procedure proposed by Mallory, which in its original form called for the dyes, anilin blue, orange G, and acid fuchsin; its important feature is mordanting in phosphotungstic acid before applying the final staining fluid. It has been variously modified in recent years and the variants are usually called modifications of the "Mallory anilin blue connective tissue stain", although some of them omit the anilin blue entirely. Among connective tissue stains are also included a variety of double, triple, and even quadruple stains of a miscellaneous nature, and the well-known Mallory phosphotungstic acid hematoxylin method, in which such strong polychrome properties of the dye are developed that no counterstain is necessary to secure proper differentiation of tissue.

A third important group of methods in animal microtechnic includes those for *neurological staining.* These methods are very numerous and all highly specialized. Many of them (as is true of some methods for demonstrating connective tissue) are not actually dyeing procedures, but depend on the use of silver salts with which tissue elements are impregnated so that the desired structures are made visible when the salts are converted into metallic silver. As these silver methods do not involve dyes, no attention is given to them in the following pages. (They are, however, described in Staining Procedures,* leaflets I-B and I-C.) This is not to say, however, that dyes are not used by neurologists in microtechnic; many such methods, as a matter of fact, do call for dyes, either with or without impregnation of the tissue with metallic salts.

In addition to these three groups of procedures in animal histology, there are stains for other types of tissue and body fluids. Fats, for example, are stained by special oil soluble dyes, such as Sudan IV. Blood has its own special methods of staining, most of which depend on the use of compound stains, prepared by allowing eosin or a related dye to react upon a mixture of dyes of the methylene blue group; such compound dyes are also useful in staining bone marrow. Bone has its own special methods, many of which depend on the use of alizarin red S, which is particularly valuable for demonstrating bone in small specimens that have been cleared by treatment in alkali.

Histochemistry. Many of the above-mentioned specialized staining methods come close to yielding actual chemical information as to the nature of cell constituents. Some theories of staining do postulate chemical affinities to explain differential staining; but they do not go very far in the matter, chiefly because stains are ordinarily applied to fixed tissue, whose chemical nature is realized to be very different from that which occurs in nature. The introduction, however, of rapid freezing methods is now enabling histochemists to get away from chemical fixation and is giving them material for study which is more nearly representative of natural conditions. Also their study of enzymes on the one hand, and their identification of the individual nucleic acids on the other are gradually giving them criteria to show which stains or other reagents are actually specific for definite cytochemical ingredients. Especially important has become the use of the Schiff reagent (fuchsin-sulphurous acid), after some oxidizing agent, in the identification of mucopolysaccharides. Developments in histochemistry are now becoming so rapid that predictions are hard to make. Whether the histochemical reagents of the future will be primarily dyes or other chemicals, it is impossible to say at present.

Plant Histology. A rough grouping of the most common plant histological methods can be made by recognizing general tissue

*Conn and Darrow (1943–52).

stains and stains for woody tissue. This grouping is no more absolute, however, than the separation between general tissue stains and connective tissue stains in the zoological field. Probably the most frequently used dye in botanical microtechnic is hematoxylin, which is employed in a number of procedures either for general tissue or woody tissue. Another very valuable dye is safranin which is one of the best nuclear stains employed in botanical work; with a green or blue counterstain, very effective pictures are obtained.

Various combinations of stains have been employed for certain more highly specialized purposes, such as staining fungi in tissues, or for demonstrating pollen tubes in the style. For such purposes as this, numerous dyes have been called for, such as thionin, orange G, martius yellow, malachite green, acid fuchsin, methyl green, carmine, basic fuchsin, and lacmoid. It is nevertheless true, as stated above, that there is by no means such a variety of staining procedures in plant histology as in the zoological field.

Plant Cytology. Although the statement just made is true in the general histological field, it cannot be made in regard to cytology. The earliest cytological work was done with plant material, probably because of the greater ease with which rapidly dividing cells (meristematic tissue) can be obtained from plants than from animals. The result is a very considerable variety of procedures in plant cytology. Best known among them, undoubtedly, are: the variations of the hematoxylin methods; the Flemming triple stain, which calls for crystal violet, safranin, and orange G; and the aceto-carmine method employed for fresh smears of anthers. There are numerous special combinations of crystal violet and of safranin, each with particular counterstains designed to bring out certain desired effects.

It is interesting to note that, although there are many dyes used in both plant and animal microtechnic, there are others that are important in one of these fields but not in the other. Thus carmine and safranin are pre-eminently botanical stains, methylene blue a very important zoological stain that is hardly ever employed by the botanist.

Microbiology. Another very important field in which biological stains are employed is the study of bacteria and related microorganisms. The dyes thus used are somewhat limited in number, methylene blue, crystal violet and basic fuchsin satisfying most of the bacteriologists' needs. It is very interesting, however, to note that these three dyes, which are pre-eminently bacteriological stains, are by far the largest sellers of any of the biologicals. This indicates that although the bacteriologist does not employ as many staining procedures as the worker in other fields, he is by far the most important customer, in point of quantity ordered, of any to whom the manufacturer supplies these products.

A comparatively new field is now *bacterial cytology*. As in the case of histochemistry, development in this field has been made possible by improved methods of fixation. The conventional method of rendering bacterial cells stainable was merely to dry them on a slide, a process which distorts the cell and makes internal structure quite difficult to demonstrate. Such crude fixation methods are now being replaced, for all delicate work, with others that cause less distortion; and gradually bacterial cytology is being revealed. There are difficulties still to be surmounted; artefacts and true nuclear structures are sometimes difficult to tell apart, and misinterpretations are frequently made. Nevertheless, the field is being rapidly explored, and the subject of bacterial cytology is no longer so highly speculative as it was a comparatively few years ago.

Bacteriological staining methods (outside of cytology) can be divided into two groups: stains for bacteria in dried films; and stains for bacteria in tissue. The former consist of very simple procedures, ordinarily; about the most complicated of them is the Gram stain which calls for crystal violet and a counterstain; some species of bacteria taking the violet stain, others, the counterstain. The stains for bacteria in tissue are essentially histological methods, many of them very similar to those used for general animal tissue; their main purpose, however, is not the differentiation between different tissue elements but that between bacteria and the tissue in which they are located.

An important aspect of bacterial staining is the laboratory diagnosis of disease. In numerous diseases, of which tuberculosis and diphtheria are the most conspicuous examples, staining procedures play a prominent role in diagnosis. So important is this use that in spite of the smaller number of staining methods employed by the bacteriologist, he orders stains by the kilo while the zoologist may content himself with 10-gram bottles. This is the real reason why the bacteriologist is the chief customer of the biological stain company.

BIOLOGICAL STAINS IN TIME OF WAR

The last mentioned use of stains has made them a very important war commodity. The first World War proved this. Before that war practically all dyes had come from Germany, and no biological stains were considered reliable unless they bore the label of one particular German company. In 1914, the blockade of Germany prevented its dyes from going overseas and biologists in many other countries began to wonder where they would get fresh supplies of stains when the stocks of German dyes on hand at the beginning of hostilities were exhausted. In America this did not prove an immediate problem, for those stocks were quite large, partly in the hands of the users and partly in the store-

rooms of dealers, and if the demand had been only that of peacetime, probably the situation would not have become acute for several years. As soon as this country became involved in the war, however, the demand greatly increased. Every Army or Navy hospital had to have its laboratory; and because of the higher degree of medical care given the men than in any previous war, such hospitals and laboratories soon became quite numerous. The demand for stains, therefore, broke all previous precedents and the stock of German dyes available was no longer anywhere near enough for the demand. Laboratory technicians soon were forced to get familiar with American dyes—and they learned, in the hard way, that biological stains need standardization.

After that war the demand for stains seems to have decreased, although no actual figures are available. Then, gradually, during the next 20 years, the demand increased as new laboratories were started and new uses for stains proposed, but it did not reach a very high figure until 1939. Then almost simultaneously with the German invasion of Poland, the first marked increase in the demand (since the previous war) was noted. Just why the increase occurred then in America is hard to figure; but it is possible some users of dyes became panicky for fear that the coming of war would cause shortages in this field. No such shortage did develop, in America, at least; and it is well that it did not, for as soon as the U. S. Government, through Army, Navy, and Lend-Lease agencies, began ordering stains on the war basis, the increase in orders exceeded anything that had been previously thought possible. By 1943 the demand for stains was fully ten times that of 1939. It seems almost incredible to believe that any Government in war should find it necessary to order any dye (even such a dye as crystal violet) by the thousands of bottles; and the fact that such orders were received again and again gives some idea as to the number of field hospitals called for in modern warfare.

COMMERCIAL SOURCES OF STAINS

The early biologists naturally obtained their dyes from concerns whose primary business was supplying such products to the textile industry. This proved unsatisfactory because of the great variation in the dyes thus obtained, even though sold under the same name. To improve the situation, Dr. Weigert, in 1880, advised a Dr. Georg Grübler, a student of Ludwig and Dreschel at Leipzig, to concern himself with marketing anilin dyes especially to use in microscopy. Dr. Grübler, accordingly, founded a company (first registered as "Physiol.-chemisches Laboratorium, Dr. Georg Grübler"; subsequently known as "Dr. G. Grübler & Company") which originally dealt with stains and other physiologico-chemical preparations, but later specialized almost entirely in dyes.

In 1896 Dr. Grübler had to withdraw from this firm and a former partner of Dr. Grübler's named Schmid, became the proprietor. After retiring from this business, Dr. Grübler started a laboratory of his own which continued for some time to supply Dr. G. Grübler & Company with certain products. This laboratory was subsequently incorporated in 1897 under the name of "Dr. G. Grübler's mikroskopisch-, chemisch-bacteriologisches Laboratorium," and was sold to Dr. Hollborn. The company thus founded subsequently became "Dr. K. Hollborn & Söhne", and for a number of years continued its relations to the Grübler company. It did not, however, supply the latter company after 1921, but began marketing such products on its own account.

This matter is gone into in some detail because from 1921 till about 1945 there were two rival firms producing stains in Germany, the Dr. G. Grübler & Company and K. Hollborn & Sons; and the relationship between these two concerns is not generally understood. Each company made certain claims that are often interpreted as implying that it produced the only genuine Grübler stains. As a matter of fact, neither company manufactured dyes and the actual claims of the two companies were these: G. Grübler & Company claimed that they alone had the list of commercial sources from which Dr. Grübler obtained his dyes some 40 years previously and the standards set up by him at that time; K. Hollborn & Sons claimed that they were the only concern to produce the so-called "original Grübler preparations", these preparations being certain staining fluids and combinations of stains (e.g., the Giemsa stain) first developed in Dr. Grübler's private laboratory. It will be seen that these claims thus stated were not contradictory, but misunderstanding sometimes resulted from the advertisements published by the two companies.

The Grübler stains were pretty generally used throughout the world until about 1916–18. The first World War changed the situation, because the temporary unavailability of German stains forced England and the United States to develop their own sources of stains. The second World War had an even greater effect on the situation; and some time either during the war or just after, the two above-mentioned German concerns seem to have gone out of business. There are still German stains produced, notably by E. Merck and Co., and the Bayer Co., and they are sold at prices with which American stains cannot compete in the world market; but they hardly appear on the American market now and are excluded from that of Great Britain, both of which countries now have well established stain producers of their own.

It required some time after the first World War before the biological stain production in other countries besides Germany could acquire the prestige formerly belonging to the productions of other countries. At first perhaps, there was some reason for the unfavorable reputation attained by non-German stains. It naturally required some time for the concerns just then entering the field to learn to produce stains capable of giving the same results as those obtained by the German product. As soon as the companies had solved these problems, however, their products in general were superior to those available before the war. In certain quarters the prestige of the German stains still worked to the discredit of the products of other countries; but before the second World War began, the resulting prejudice had just about come to an end. Although this prejudice exists no longer, special atten-

tion must be given here to the situation which existed in the United States and Canada, following the First World War, as it was this which led to the establishment of the Biological Stain Commission, the sponsor of this book.

MODERN STANDARDIZATION OF STAINS

The Biological Stain Commission is concerned with the inspection and standardization of stains, not with their manufacture, as is sometimes supposed. It was learned in 1920, while the post-war embargo on dyes was still in effect, that American scientists were being supplied with dyes from three or four different stain companies and that their products were not sufficiently uniform to be reliable. Accordingly, through the coöperation of the National Research Council of the United States and three or four American scientific societies, the Commission on Standardization of Biological Stains (now known as the Biological Stain Commission) was established. The Commission is now an independent non-profit corporation, having representatives on its membership of eight American scientific societies with which it coöperates. The work of the Commission is two-fold. First, by coöperation of various biologists and chemists it gathers information concerning the nature of dyes as related to their use in microscopic technic; secondly, by working with the manufacturers and dealers it endeavors to see that the supply of stains available in America is of the highest possible quality as judged by their performance in actual laboratory use. The first of these purposes has inspired this book, which is now in its sixth edition, and at the same time has led to the publication by the Commission of a journal, Stain Technology and a loose-leaf manual, Staining Procedures (Conn and Darrow, 1943–4). The second object is being brought about by the plan of certifying stains.

The certification plan has been adopted because of the great difficulty of drawing up any chemical or physical standards to determine which stains are satisfactory and which are not. If such standards could be formulated, it would be possible to draw up specifications with which manufacturers of stains would be expected to comply.

Specifications. Drawing up specifications for biological stains has proved difficult. In many cases their chemistry is not entirely understood, and even when it is, their behavior in actual use is often dependent upon the amount and nature of impurities that may be present in only minute quantities. Since these impurities are sometimes beneficial and sometimes detrimental and their nature is often unknown, practical specifications are much more difficult to draw up than in the case of ordinary chemicals where a high degree of purity can be obtained and any impurity is undesirable.

Nearly 30 years ago the Stain Commission began trying to draw up specifications for some of the most commonly used stains. These specifications were published in the first edition of this book. They did not prove very useful, however, and of recent years no great attention has been paid to them. Much more workable specifications have been secured during the last five years with the coöperation of the National Formulary Committee of the American Pharmaceutical Association.

In several recent editions of the National Formulary, published by the American Pharmaceutical Association, a section has been included in which formulae of staining solutions are given. Originally there was no agreement between these formulae and the ones recommended by the Stain Commission. Beginning in 1937, however, it was decided that the National Formulary Committee and the Commission on Standardization of Biological Stains should coöperate in this matter. As a result of this cooperation there have been several worth-while accomplishments:

1. Specifications of the most important stains now on the certification basis have been drawn up and have been published in recent editions of the National Formulary. These specifications are partly chemical and spectrophotometric, but also contain detailed statements as to how the stains should be tested as to their behavior for biological purposes and state the results to be expected from these tests. In every case these specifications have been made to harmonize with the tests as actually performed by the Stain Commission.

2. The formulae of staining solutions given in the National Formulary, in STAINING PROCEDURES, and in the Manual of Methods for Pure Culture Study of Bacteria, published by the Society of American Bacteriologists, have been compared and studied critically with the object of publishing identical formulae in all three places. This has resulted in a few changes from the formulae given in early editions of this book.

3. Another result of this coöperation has been to give the work of the Stain Commission more of an official standing than it had before. The Stain Commission was originally formed as an entirely unofficial organization and has never had any connection with any government organization. The specifications of the United States Pharmacopoeia and the National Formulary, however, do have official recognition. The publication of the seventh edition of the Formulary, therefore, with its references to stains certified by the Commission, has resulted in their official adoption for many purposes throughout the United States. Most notable has been the adoption by the United States Armed Services in connection with purchases made by their procurement agencies. Inasmuch as the seventh edition of the National Formulary was published at about

the time the United States entered the second World War, this proved a development of considerable significance.

4. It has been realized for some time that the specifications would be more satisfactory if they could eliminate statements that the dye under consideration should be satisfactory for such and such procedures. This has proved difficult because of lack of correlation between chemical and optical characteristics, on the one hand, and performance on the other. Of recent years appreciation has been growing of the fact that this lack of correlation might be due to insufficient data; and that the only way to find out would be to collect the data. Accordingly the assay laboratory of the Stain Commission has been going over its collection of stain samples, some satisfactory and some unsatisfactory, to see if any agreement between the two types of specification can be found. It proves that such agreement is lacking in so many cases that specifications based on performance must still be included; but the data that are being collected are nevertheless proving distinctly valuable. They are showing better than ever before just what limits of variation in chemical and physical specifications are permissible; and as a result it is now possible to establish better definitions of and specifications for biological stains than ever before. Much of this new information is being incorporated into this edition of Biological Stains.

Standardization Procedures now in Operation. This interrelation between the National Formulary specifications and the standardization methods of the Stain Commission makes these methods of considerable interest. It must be explained that the standardization procedures adopted by the Stain Commission have been entirely on the batch basis—that is, approval is not given to the entire product of any one company, but is extended to each individual batch that is put on the market. The reason for this is that the Commission has never felt willing to place reliance on chemical or optical tests. All the specifications that have been drawn up by the Stain Commission or by the National Formulary Committee have contained statements which mean essentially as follows: "The sample must prove satisfactory when tested by the following procedures: - - - - -". It is obvious that until it is known just how the results obtained in practical use correlate with chemical or optical properties, any tests depending upon performance must be carried out for each individual batch.

Commission Certified stains are now coming to be designated with the initials "C.C." following the name of the dye.

There is no need of listing here the tests actually employed by the Commission; they are given in an appendix of this book where they can be consulted by anyone who is interested. It must, however, be explained that these tests fall into two groups: chemical and optical on the one hand; tests for performance on the other.

In interpreting results, greatest weight is given to the tests for performance. It not infrequently happens that a batch of some stain will prove to give perfectly satisfactory results in all the latter, but proves slightly low in dye content, or disagrees in some respect with optical characteristics called for in the specifications. The rule in the case of such a sample is to approve it on the basis of its performance in spite of the slight non-conformity in the case of chemical or optical characteristics.

Batches of dyes thus approved by the Commission are sold by the companies with a special label furnished by the Commission, known as the certification label. This label indicates the certification number of the batch and is not supposed to be employed for any batch other than that on which it is issued. A certification label on any bottle of stain means therefore, five things: (1) a sample of the batch bearing the label has been submitted to the Commission for testing and a portion of the sample is permanently on file; (2) the sample proves true to type, as judged by spectrophotometric tests; (3) its dye content is up to specification and is correctly indicated on the label; (4) it has been tested by experts in the procedures named on the label and has been found satisfactory by them; and lastly, (5) no other batch can be sold under the same certification number except by such a flagrant breach of confidence on the part of the company as to risk losing the good will of the Commission.

At the present time (1952), 51 stains have been put on the certification basis. These include all the most commonly used biological stains as well as a few in which the Stain Commission has become interested, although they are not so extensively employed in the laboratory as most of the others on the list.

There are, at the time this sixth edition goes to press, nine companies in the United States and one in Canada submitting their stains to the Commission for certification before putting them on the market. It must be realized, however, that no one of these concerns necessarily manufactures all the stains which it thus submits; but in the case of any stain which is manufactured elsewhere, the company takes responsibility for its performance as a biological stain on the basis of tests made to show its adequacy, and in many instances carries out a certain degree of purification or other processing before putting the stain on the market. One of these companies puts on the market every stain now on the certification list. Three other companies submit samples of over half the stains thus listed, while the other companies merely request certification of one or two dyes in which they specialize. No dyes have yet been certified by the Stain Commission submitted by any concern outside North America. The reason for this is because of the difficulty in handling the certification of stains on the batch basis with a concern that is located at a dis-

tance, doing business in this country only through agents who are not in direct touch with the actual manufacturers of the dyes.

Although this phase of the work of the Stain Commission is one of inspection, it has not brought about unpleasant relations with any manufacturer or dealer in stains. As a matter of fact, the hearty coöperation of the American stain companies has been obtained throughout; and without this coöperation much that has been accomplished in the way of standardization would have been impossible.

The British Biological Stains Commission. Late in 1949 an organization of this name was established in England with objects similar to those of the American Commission. The situation in England is different from that in the United States, and has to be handled differently. Foreign dyes have been definitely excluded from the country for some time, and a few English concerns are producing biological stains, without much control of their products. It is still too soon to tell what the results will be of the English effort to standardize their supply. They are coöperating with the Biological Stain Commission in America, and samples of British stains have been sent to this country for testing in the Commission's laboratories. Many of the British samples submitted do not conform to the standards adopted in the U. S.; but there is little doubt but that the quality of the products will be improved now that attention is being given to the matter by a group of scientists who use them.

CHAPTER II

THE GENERAL NATURE OF DYES AND THEIR CLASSIFICATION

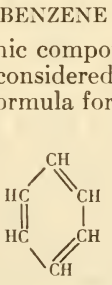
DYES are generally classed in two groups, the natural and the artificial. The former class is now of relatively smaller importance from the standpoint of the manufacturer and the textile dyer; for the artificial dyes far outnumber them and the advancement of science is gradually making it possible to produce many of the formerly natural dyes by artificial means. It just happens that one or two natural dyes, the derivatives of cochineal and logwood extract (see Chap. IX), are among the most valuable biological stains; but the natural dyes in general are so few in number than they can be practically disregarded in considering the general chemical nature of dyes.

Because the first artificial dyes were produced from anilin, all of this class are often called "anilin dyes," although there are now a large number of them which bear no relation to this compound and are not derived from it. Therefore the term is now quite largely being replaced by the more correct expression "coal-tar dyes," since all of them are made by chemical transformations from one or more substances found in coal-tar.

Comparatively little is known concerning the chemistry of the natural dyes; but the synthetic dyes have been carefully studied and volumes have been written concerning their chemical composition and its relation to their behavior. Much of this may well be ignored by the biologist who employs dyes merely for staining microscopic objects. A certain familiarity with the general principles is useful, however, in helping him to employ stains scientifically. This is particularly true now that there is a growing tendency to use dyes in biological work as histochemical reagents. The brief discussion that follows is intended to help the biologist to understand merely the fundamental principles of dye chemistry.

BENZENE

All coal-tar dyes are organic compounds of the aromatic series. In other words they may be considered as derivatives of the hydrocarbon, benzene, C_6H_6, the formula for which is usually considered to be

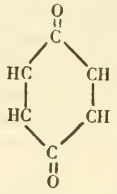


and is usually written in abbreviated form

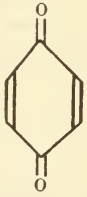
The double bonds in benzene are believed not to be fixed but to oscillate between adjacent carbon atoms.

The importance of benzene in organic chemistry is because of the infinite number of ways in which it can combine with other radicals and elements, forming compounds of extreme complexity. If the elements in such compounds are combined in certain ways the substance is colored, and such colored compounds, after slight additional changes in the molecule, become dyes. In this respect the colored benzene derivatives are unlike the colored pigments of simpler composition; many of the latter may show intense color, but do not act as dyes.

One type of substitution in the benzene ring is specially important in dye chemistry. Two atoms or groups having two valency bonds instead of one may also replace two hydrogen atoms, provided the replacement takes place simultaneously and the hydrogen atoms replaced are situated either in the ortho or in the para position to each other. Thus two oxygen atoms (which are bivalent) may replace two hydrogen atoms (which are monovalent) forming the compound known as quinone $C_6H_4O_2$, the formula for which is



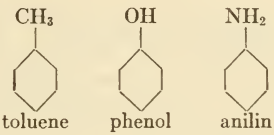
or as commonly written



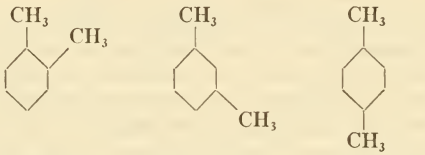
In printed formulae, such as those that follow in this book, the quinone ring is often abbreviated still further by omitting the double bonds within the ring. The substituent atoms or groups may or may not be alike, so long as both have two valency bonds entering into the combination. This type of substitution involves a rearranging of the double valency bonds in the benzene ring; and in compounds of this type, called quinoid compounds, the double bonds are supposed to be fixed, not mobile as in benzene. This

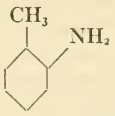
change of the valency bonds takes place very readily in many dyes, and certain peculiarities of their behavior are explained by it; (see for example p. 199).

Three mono-substitution products of benzene are of importance in considering the structure of dyes, namely; toluene or methyl-benzene, $C_6H_5 \cdot CH_3$; phenol, carbolic acid or phenylic acid, $C_6H_5 \cdot OH$; and anilin or phenyl amine, $C_6H_5 \cdot NH_2$. Their constitutional formulae are as follows:



Two important di-substitution products are xylene or dimethyl benzene $C_6H_4 \cdot (CH_3)_2$, and toluidine, $C_6H_4 \cdot CH_3 \cdot NH_2$. Both of these occur in the above mentioned three isomeric forms, as shown below for xylene:





RELATION OF MOLECULAR STRUCTURE TO COLOR

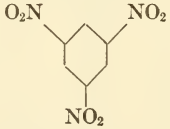
Although there is still much to learn as to the explanation of color in terms of structural formulae, it is now well known that certain definite atomic groupings (known as *chromophores*) are associated with color. The basic groupings involved in these chromophores are C:C, C:O, C:S, C:N, N:N, N:O, and NO_2; and the more of these that occur in the same compound the more pronounced the color. It will be noticed that the quinone ring, as pictured above, contains the following chromophore grouping in duplicate :C–C:C–C: . Quinone, itself, is in fact colored, and the quinone ring is one of the most important chromophores known, all compounds containing it showing intense color.

Probably if the whole situation were understood, physical chemistry could give us an explanation of why some compounds are

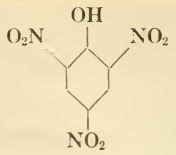
colored. We know that color is caused by selective absorption of certain wave lengths of light, so that the light transmitted by or reflected from a given substance lacks these particular wave lengths of the visible spectrum and accordingly appears colored. Such a substance, in other words, acts as if it were a prism provided with a screen to block off certain parts of the spectrum in which the refracted light is normally transmitted. Conceivably the chromophores confer upon the molecule properties of just this sort, acting possibly as a system of resonators tuned to vibrate at the same rate as the waves to be absorbed. The theory cannot be discussed here in any detail; but it should be pointed out that valence electrons under certain circumstances confer resonance on a given compound. The unsaturated compounds, such as those containing the double bonds of the chromophores, show a special tendency toward resonance; and it is a fact that as the general degree of unsaturation and complexity of a compound increases, the spectral absorption tends to pass from ultraviolet toward infra-red, that is, the color transmitted (or reflected) changes from yellow through reds and blues.

The benzene compounds containing chromophore radicals are known as chromogens. A chromogen, however, although it is colored, is not a dye, in that it possesses no affinity for fibers or tissues. It may coat them, but only mechanically, and it will be easily removed by mechanical processes. That is, it will not "take". (See, however, the discussion of fat stains, p. 69). In order for a substance to be a dye, it must contain in addition to the chromophore group, a group which imparts to the compound the property of electrolytic dissociation. Such auxiliary groups are known as auxochromes. They may slightly alter the shade of the dye, but are not the cause of the color. Their function is to furnish salt-forming properties to the compound. Certain chromophoric groups have also slight auxochromatic properties.

To illustrate these different types of groups, let us take a typical example. The nitro group ($-NO_2$) is a chromophore. When three of these groups displace three hydrogen atoms in a benzene molecule, we have the compound trinitrobenzene,



which is yellow. It is not a dye, however, but is a chromogen. It is insoluble in water, and is neither an acid nor a base; that is, it does not dissociate electrolytically and consequently cannot form salts with either alkalies or acids. If, however, one more hydrogen atom is replaced, this time with the hydroxyl group (-OH), which is an auxochrome, the resulting compound,



is an acid, capable of electrolytic dissociation and of forming salts with alkalies. It is the familiar substance picric acid, and is a yellow dye.

It will thus be seen that the color of picric acid is due to the chromophoric nitro groups, and that its dyeing properties are due to the auxochromic hydroxyl group. If the nitro groups be reduced to amino groups ($-NH_2$), which are not chromophores, the resulting compound is colorless and hence is not a dye.

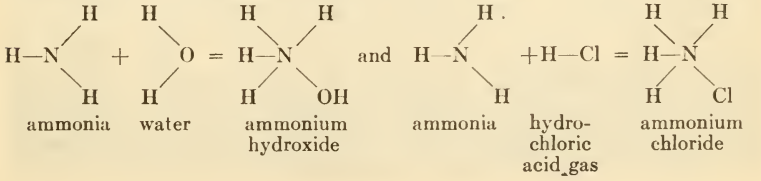
Summing up, we arrive at the definition of a dye as an organic compound which contains chromophoric and auxochromic groups attached to benzene rings, the color being attributable to the chromophores and the dyeing property to the salt-forming auxochromes.

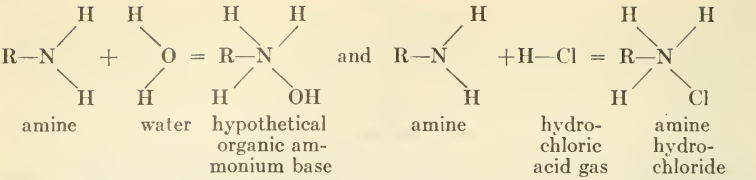
ACID AND BASIC DYES

There is frequent misunderstanding among biologists as to the meaning of the terms "acid dyes" and "basic dyes". It is sometimes assumed that the terms refer to the H-ion concentration of the dye solutions. Nothing could be further from the truth. The distinction actually depends on whether the significant part of the dye is anionic or cationic, and bears no direct relation to the reaction of any solution of the dye. As a matter of fact, some of the most strongly anionic or *acid* dyes, like eosin Y, regularly form salts with strong metallic ions such as sodium; and the greater strength of the metallic cation determines the reaction of the solution, i.e. the solution is ordinarily basic in reaction.

As the auxochromes are the salt forming groups of dyes, it is ordinarily the nature of the auxochrome present that determines in which of these two classes a dye belongs.

Some auxochromes are basic, e.g., the amino group ($-NH_2$), while others are acidic, e.g., the carbonyl group (-COOH). The amino group owes its basic character (which it transmits to the whole molecule) to the ability of its nitrogen atom to become pentavalent by the addition of the elements of water (or of an acid), just as in the case of ammonia; thus:





In other words, the NH_2-group yields hydroxyl ions and enables the compound to ionize and to act as a cation in forming salts; such a dye is a basic dye.

The carboxyl group (-COOH), on the other hand, is acidic, as it can furnish hydrogen ions by electolytic dissociation. Many of the most important acid dyes contain this group; as they ordinarily occur as sodium salts, the characteristic group in these dyes is -COONa. Other acid dyes contain the rather weaker auxochrome group -OH, and form salts in -ONa.

The more of one of these groups in a compound, the stronger base or acid it becomes. If there is one of each, the basic character of the amino group predominates, but is weakened by the influence of the acidic group. The strength of both groups is also influenced by other groups or atoms in the compound; thus, for example, the chromophore $-NO_2$, although incapable in itself of conferring acid properties to the compound, exerts an influence to make any hydroxyl group in the compound more strongly acidic, in other words to become more highly dissociated electrolytically.

One other group of atoms encountered in dye chemistry needs explanation, namely the sulfonic group, $-SO_3H$. It is a salt-forming group of strongly acidic character, in that it suffers extensive electrolytic dissociation. This group, however, is only very feebly auxochromic. Its function is to render a dye soluble in water, or to change an otherwise basic dye into an acidic one, as in the case of the fuchsins, where the strongly basic "fuchsins" are changed into the strongly acid "acid fuchsins" merely by the introduction of sulfonic groups into the former. A compound which contains a chromophore group and a sulfonic group is not a dye, however, unless there is also present a true auxochrome group.

From this it can be understood what is meant by calling dyes either basic or acidic. It neither indicates reaction nor does it mean that the dyes of commerce are actually bases or acids. They are ordinarily salts. An acid dye is a salt of a color acid—usually its sodium salt, but occasionally a salt of potassium, calcium or ammonium. A basic dye is a salt of a color base—usually a chloride, but sometimes a sulfate or acetate. (The oil-soluble dyes present an exception to this statement; see p. 76.) The terms *anionic* and *cationic dyes* would be more suitable, but they have never come into general usage. Basic dyes are sometimes available as free bases, in which case the name is ordinarily followed by

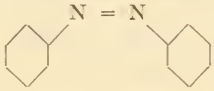
the word "base". Thus "basic fuchsin" means the salt of fuchsin with some colorless acid (e.g., hydrochloric) while "fuchsin base" indicates the color base of this dye, not combined with any acid.

THE CHROMOPHORES

As stated above, every dye contains at least one group of atoms known as a chromophore, which is regarded as being responsible for the colored properties of the compounds in which it occurs. Some of these chromophores have a basic character, others acid. There are only a comparatively small number of them which enter into the usual biological stains, and only these need be considered here. They are as follows:

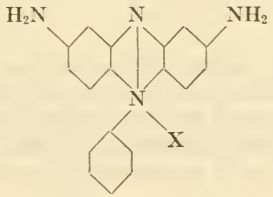
BASIC CHROMOPHORES

1. *The azo group,*—N=N—, which is found in all azo dyes, of which methyl orange and Bismarck brown are well known examples. In all these dyes, a benzene ring is attached to each nitrogen atom. All the dyes of this group may be looked upon as derivatives of azobenzene,



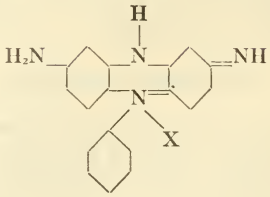
2. *The azin group,*

which is found in phenazins, of which neutral red and the safranins are good representatives. The skeleton formula of a safranin is:

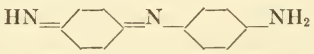


in which x represents the negative ion of a monobasic acid such as hydrochloric, acetic, nitric or sulfuric. This chromophore is capable of a variety of rearrangements of its valency bonds, as the bond between the two nitrogen atoms may disappear and the com-

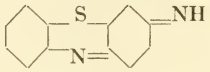
pound assume a quinoid structure, as for example the following grouping:



3. *The indamin group*, —N=, as observed in the indamins, thiazins, and so forth. Methylene blue is the best known representative of this group. In these dyes, two benzene rings are attached to the nitrogen atom, one of these being in the quinoid form and hence adding a second chromophore. The typical indamin formula is:



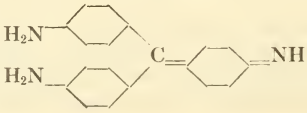
In the thiazins, such as methylene blue, the two benzene rings are further joined together by a sulfur atom, forming three closed rings of atoms. The simplest thiazin base would be:



ACID CHROMOPHORES

1. *The nitro group*, —NO_2, as in picric acid
2. *The quinoid benzene ring*,

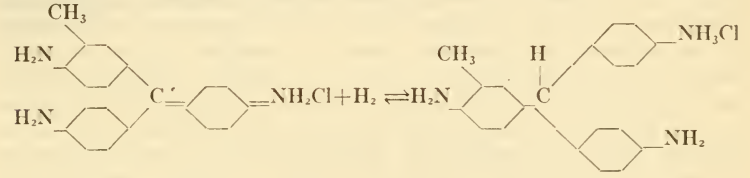
which occurs in a long series of dyes, such as the indamins above mentioned, the xanthenes and the di- and tri-phenyl methanes, which include many well known stains, such as rosolic acid, fuchsin, methyl green and the methyl violets. A typical triphenyl methane formula is that of pararosanilin base:



LEUCO COMPOUNDS

The different chromophores differ considerably from one another, but they all have one property in common. In the language of chemistry, they all have unsatisfied affinities for hydrogen; or in other words, they are all easily reducible, for combining with hydrogen is the opposite of oxidation and is, therefore, reduction. The nitro group may be reduced to an amino group; in the azin group the bond between the nitrogen atoms may break and two hydrogen atoms be taken on; while in the various chromophores with double bonds (such as the quinoid ring) the double bond may break and hydrogen atoms become attached to the valencies thus freed.

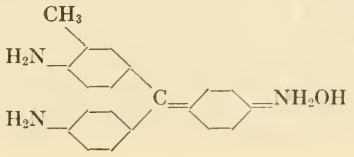
Now in every case this reduction destroys the chromophore group, and as a result the compound loses its color. In other words a dye retains its color only as long as its affinities for hydrogen are not completely satisfied. These colorless compounds are known as leuco compounds; thus fuchsin yields leuco-fuchsin on reduction, and methylene blue reduces to leuco-methylene-blue. For example:



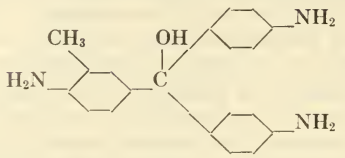
fuchsin leuco-fuchsin

Ordinarily this reaction is reversible under conditions favoring oxidation. It is of especial significance to the bacteriologist, as dyes can often be used as indicators of reduction.

Certain dyes form a still different type of leuco compound, often called a "leuco-base." We have seen that the basic dyes ordinarily occur as salts of some colorless acid; now, in the case of certain dyes, notably the tri-phenyl methanes and xanthenes (Chapters VII and VIII), as soon as the acid radical is removed, the compound becomes colorless. This is because a rearrangement of the atoms in the molecule takes place upon neutralization so as to give, not the true dye base, but a compound known as a carbinol (see p. 137) in which the chromophore does not occur. Thus the theoretical base of fuchsin which should be obtained upon removal of the acid radical is:



The compound actually formed, however, is the pseudo-base or carbinol:



In this compound, it will be readily seen, there is no chromophore; hence it is colorless. These pseudo-bases are of little significance to the biologist, but they are of importance to the dye manufacturer as intermediates in the preparation of dyes.

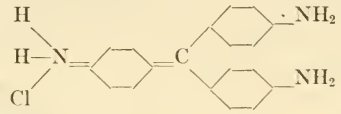
In the case of many acid dyes the chromophore is similarly broken by a rearrangement of the atoms which occurs on neutralization. This reaction is ordinarily very readily reversible and makes such dyes useful indicators of acidity. It is discussed more fully under acid fuchsin (p. 154) and phenolphthalein (p. 198).

In the case of basic fuchsin, a still different type of leuco compound can be obtained, which has recently acquired considerable importance, both in cytology and in histochemistry. When this dye is reduced with sulfurous acid or a sulfite, a type of leucofuchsin is produced which is known as Schiff's reagent, and has been used by chemists for many years as an indicator of the presence of aldehyde. Originally this reagent was thought to be no different from the leucofuchsin referred to on the preceding page; but when its use as a histochemical reagent was first introduced it was noticed that the restored color, after contact with the aldehyde-like constituents of cells, was somewhat violet, rather than red. This seemed to indicate that some other chemical change in the dye takes place in addition to the reduction. It is now believed that the sulfite radical combines with the reduced compound in some way; and the resulting compound, Schiff's reagent, is now generally called fuchsin-sulfurous acid, rather than leuco-fuchsin. The importance of this reagent is being appreciated more and more, as histochemistry increases in significance; and it will be treated more fully elsewhere in this book.

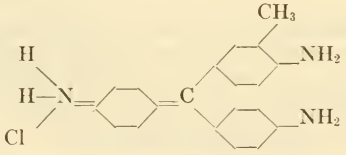
CLASSIFICATION OF DYES

On the basis of the chromophore present the simple synthetic dyes are classified into several groups. If each of these groups were characterized by a single color or by a few closely related colors, dye chemistry would be a comparatively simple proposition. As a matter of fact a single chromophore may occur in dyes of practically all colors of the rainbow. It is ordinarily impossible to determine, *a priori*, from the chemical formula of a dye what particular color the compound may have; but there is, nevertheless, a

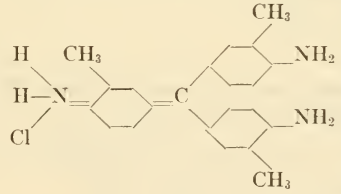
certain general rule which correlates chemical formula with color. In any group of compounds, the simpler ones are converted into the more complex by substitution of radicals for hydrogen atoms. In the dyes the substituents are generally methyl or ethyl groups, or sometimes phenyl groups. Now the general rule is that the larger the number of hydrogen atoms that have been replaced by these groups the deeper the color. The tendency is for the color of the simplest dyes in any group of homologous compounds to be yellow, passing through red to violets and then greens and blues, as the homologs become higher through the introduction of successively larger numbers of methyl or other substituting groups. Thus the compound pararosanilin, which is very frequently sold as basic fuchsin is a triphenyl methane, with an amino group attached to each benzene ring, but without any methyl groups; thus:



Rosanilin, which is similar in composition, but contains one methyl group attached to one of the benzene rings,



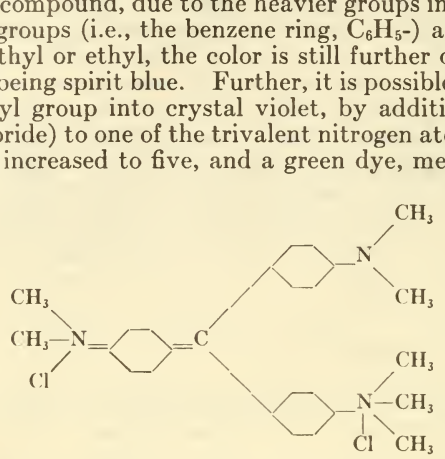
is a red very similar to pararosanilin but with less of a yellowish cast. Now another methyl group may be introduced into each of the other two benzene rings, and each one successively deepens the shade of red, so that the highest homolog of the series, new fuchsin:



has a more bluish cast than any of the others. Thus basic fuchsins can vary considerably in their shade according to the proportions in which these four possible components may be mixed.

It is also possible in another way to deepen the color of pararosanilin still further, namely by introducing methyl groups into the amino radicals instead of directly on the benzene rings. Thus the methyl violets are obtained; and the more methyl groups intro-

duced the bluer the violet, until when all six available hydrogen atoms are thus substituted, crystal violet, the deepest of them all, is obtained. By using three ethyl groups instead of methyl, Hofmann's violet or dahlia is formed, which is deeper in color than the trimethyl compound, due to the heavier groups introduced. If three phenyl groups (i.e., the benzene ring, C_6H_5-) are introduced instead of methyl or ethyl, the color is still further deepened, the resulting dye being spirit blue. Further, it is possible to introduce another methyl group into crystal violet, by addition of methyl iodide (or chloride) to one of the trivalent nitrogen atoms, whereby its valency is increased to five, and a green dye, methyl green, is produced:



With these facts in mind it will be seen that the grouping of dyes as based upon these chromophores does not classify them in relation to their color. It is a useful classification, however, because it puts together those that have similar chemical structure. The important biological dyes, thus classified, fall into the following groups:

A. The nitroso dyes.
   e.g., *naphthol green B.*

B. The nitro dyes.
   e.g., *picric acid.*

C. The azo dyes.
   e.g., *methyl orange, Bismarck brown Y, orange G, Congo red, Sudan III and Sudan IV.*

D. The quinone-imine group, including
   1. Indamins; e.g. *toluylene blue.*
   2. The indophenols
   3. Thiazins; e.g., *thionin, toluidine blue, methylene blue.*
   4. Oxazins; e.g., *brilliant cresyl blue, Nile blue.*
   5. Azins, including
      (a) Amino-azins; e.g., *neutral red.*
      (b) Safranins; e.g., *safranin O, magdala red.*
      (c) Indulins; e.g., *nigrosin.*

E. The phenyl-methane dyes, including
   1. Diphenyl-methanes, e.g., *auramine O.*
   2. Diamino triphenyl methanes; e.g., *malachite green, brilliant green, light green.*
   3. Triamino triphenyl methanes; e.g., *basic fuchsin, acid fuchsin, methyl violet, gentian violet, methyl green, anilin blue.*
   4. Hydroxy triphenyl methanes (rosolic acids); e.g., *aurin, red corallin.*
   5. Diphenyl naphthyl methanes; e.g., *Victoria blue R.*

F. The xanthene dyes, including
   1. Pyronins; e.g., *pyronin Y* and *B.*
   2. Rhodamines; e.g., *rhodamine B.*
   3. Fluoran derivatives; e.g., *eosins, erythrosin, rose bengal.*
   4. Phenolphthalein and the sulphonphthaleins.
   5. The acridine dyes, e.g., *acriflavine.*

G. The anthraquinone dyes.
   e.g., *alizarin.*

H. The thiazole dyes.
   e.g., *titan yellow.*

I. The quinoline dyes.
   e.g., *pinacyanol.*

DYE NOMENCLATURE

Very little system has been used in naming dyes, and as a result their nomenclature is extremely confused. Generally the manufacturer of a dye which he thinks is new or which he wishes the public to consider a new dye sells it under a new name which is not intended to give any clue as to the nature of the dye. If the manufacturer knows that the name is a mere synonym of one already in use he does not say so, for he wishes to encourage the sale of his own product rather than that of some other dye maker. Accordingly it has been left for others, who are not financially interested, to work out the synonymy of the dyes; and the list of names that are found to apply to a single dye is sometimes amazing.

With the dyes in general so unsystematically named, it is natural that the same confusion should reign in the nomenclature of biological stains. This confusion is very unfortunate, for it often misleads the biologist as to just what he is doing. For example, some histologist may have on hand a bottle of stain labeled dahlia and he may find it useful for some new technic, which he publishes; while another may propose for an entirely different technic the

stain Hofmann's violet. Then a third laboratory worker may read both articles and wish to try both methods; so he accordingly orders both dahlia and Hofmann's violet. His dealer, who may be unacquainted with dyes, will very likely send him a bottle bearing each name, and the purchaser has no easy way of discovering that the two are identical; so he may continue for years to use the two stains for different purposes, misled by their labels and thinking them distinct. The manufacturers and dealers in stains have sometimes encouraged this confusion by their practice of taking care to have the label on the bottle agree with the name used in the customer's order, regardless as to what the usual name for the dye may be.

An attempt to relieve this confusion has been made by the Commission on Standardization of Biological Stains (1923f) by publishing a list of biological stains with their best known synonyms. In each case one of the names is listed as a preferred designation. Sometimes general usage made it easy to select one name as the preferred one; but in other instances the selection was more or less arbitrary. This same list, with a few revisions in the way of additions and corrections, is given in the appendix of this book (p. 252; see p. 106 of first edition). A few new stains have been added to this list, but essentially it is the same as published in 1923. The chief changes have been in the list of synonyms, which has been revised to omit names that are obsolete and have no present meaning. With but one or two exceptions the preferred designations are still the same as in the first list.

DYE INDEXES

There have been two important indexes of dyes, one published in Germany (Schultz' Farbstofftabellen) the other in England (the Colour Index of the Society of Dyers and Colourists). Seven editions of the former appeared up to the time of the second World War, while only one edition (1923) has yet appeared of the latter. It was natural that the earliest efforts to index dyes should have been made in Germany as there was almost no dye industry outside that country until 1914; but the 1923 edition of Schultz was so much less complete than the Colour Index which came out the same year that there has been a recent tendency to follow the latter rather than the former. In the second edition of the present book, therefore, the Colour Index numbers of the various dyes were listed instead of the Schultz numbers.

The seventh Schultz index was as complete as the Colour Index, with an entirely new system of index numbers assigned to the dyes, agreeing neither with that in the other publication nor with earlier editions of Schultz. Considering how often it is desirable to refer to a dye by an index number, it is unfortunate that these numbers cannot be standardized in some way by international agreement.

In the present edition of this book the Colour Index numbers (abbreviated C. I. No.) are still used, so as to avoid confusion and to make the present index numbers agree with the list previously published. The Schultz number of each of them is given for reference purposes, however, in the lists in the appendix, pp. 252, 260.

Although there has been as yet only one edition of the Colour Index, a new one is now in course of preparation by the Society of Dyers and Colourists in England, with the cooperation of the American Association of Textile Chemists and Colorists. The new edition is to be entirely different from the first, consisting of more than one volume; and it will contain many more dyes than ever before listed. Although work on it has been in progress for some time, no probable publication date has yet been announced, as the task is proving a large one. The compilers of this Index are fully aware of the confusion caused by frequent changes in index numbers; so it is their plan to adopt an entirely new system in the second edition, with numbers so different in appearance from the old ones that they cannot be confused with them. The idea is to have the new numbering system one that will allow for expansion anywhere and thus avoid the necessity of continual change with each new edition. If this system works out as hoped, it is possible that in the next edition of this book, the new C.I. numbers can be followed.

At the time of the fourth edition of this book, the plans for the revision of the Colour Index were not known, and it was feared that as soon as a second edition of that Index appeared, further reference to the old C. I. Numbers would be distinctly confusing. In anticipation of this situation the 4th edition of the present book included at the head of the description of the various dyes, a series of numbers designed particularly to apply to biological stains. This policy is continued in the present edition. The new numbers are printed in a smaller type than the Colour Index numbers and no special significance is being given to them. It is planned, however, to use them in the future under the designation "Biological Stain No." only if a new edition of the Colour Index makes it necessary to abandon the "C.I. Nos." at present in use without furnishing more satisfactory index numbers in their place. It is hoped that because of the relatively small number of dyes used as biological stains and the fact that the "B.S. Nos." at present selected are not entirely consecutive, there would be no need of changing the significance of each number whenever a new edition appears. Whether or not to use them cannot be decided until the new Colour Index appears.

DYE SOLUBILITIES

Textile dyes are never of a high degree of purity. Some of the impurities are accidental; others are added intentionally so that

dyers can obtain the desired shade without having to measure out dyes in very small quantities. Inasmuch as the early biological stains were textile dyes without much, if any, modification, it is natural that some of them should also have been of low dye content, and also that different batches should have been of various degrees of purity. In general the present dyes are much more pure than those available before the war of 1914–18. This makes it difficult to prepare stain solutions identical in strength with those prepared before that time.

There are two general types of stain formulae: in one a definite weight of dry dye is specified; in the other a certain volume of a saturated (generally alcoholic) solution of the dye. Each type of formula has its own possibilities of error; and to appreciate the problem it is necessary to understand certain facts in regard to the solubilities of dyes.

The error inherent in the first type of formula is plain at a glance. If two different staining solutions are made up containing 1 g. per 100 ml. of dry methylene blue, and in one case the actual dye content of the dry stain is 90 percent, while in the other only 55 percent (a difference actually observed in samples at one time on the market), it is plain that the two solutions must differ greatly in their strength. For this reason an early recommendation of the Commission (1923b) was that formulae of the second type be preferred, on the assumption that a saturated solution of a dye would be more likely to be of constant dye content than different lots of dry stain bought in the market.

This recommendation, however, was made without complete understanding of the actual facts of the case. The amount of a dye that will go into solution in either water or alcohol depends upon the amount of mineral salts present. If a dye contains a large percentage of sodium chloride, for instance, a saturated solution will be of considerably lower actual dye content than if the dye were free or nearly free from salt; the sodium chloride prevents the solvent from taking up as much of the dye as it would normally. For this reason two staining solutions each containing 10 percent by volume of a saturated solution of the two methylene blues above mentioned would be quite different from each other in actual dye content, although possibly more nearly alike than if they had been prepared with identical weights of the dry stain.

As soon as the facts were fully understood the Commission (1933c) modified its recommendation. It is plain that the only way two stain solutions can be made identical, if different batches of stain are used, is to make them up on the basis of the weight of *actual dye* present in the stain samples employed. For some time now, all staining formulae recommended by the Commission have been put in such a form as to call for a definite weight of dye of a specified dye content—thus enabling the user to recognize any

correction he has to make if he has a dye sample of different dye content. Many individual writers, however, especially writers of text books, have not followed this system. There are various reasons such a plan has not been generally adopted. The chief reason is that there is much variation in the dye content of individual batches, and it is obvious that the staining formulae cannot be generally put in the form recommended by the Commission unless the manufacturers print the actual dye content of the batch of stain on the container in which it is sold. Until about 1930 this was never done. Since about that time, however, the Stain Commission has been issuing its certification only when the dye content is printed on the label, except in instances where the necessary analytical methods are not available. This policy has gradually been changing the situation. At the present time almost all stains on the American market, except natural dyes, compound dyes like the blood stains, and a few complex dyes for which good analytical methods are still unavailable, are labeled as to their dye content. In all Commission publications (including Stain Technology) all formulae are given in terms of grams of Commission certified stains (unless the dye in question has never been certified), and as certification is now issued only on samples that vary within narrow limits, this amounts to recommending definite weights of actual dye. Gradually authors of papers published elsewhere are getting into the same habit; so the situation is much more satisfactory than a few decades ago.

In the present edition of this book there are listed, both under the individual dyes and in Table 6 in the Appendix, the solubilities of pure dyes in 95% alcohol and in water at 20° of the most commonly used stains. These data were originally published by Holmes (1927, 1928, 1929) and are based on determinations made by a coöperating laboratory of the U. S. Department of Agriculture. The author of these papers experienced considerable difficulty in obtaining the information given therein because of the strong effect of impurities upon the amount of dye capable of going into solution. The commercial samples of the dyes are rarely pure, and Holmes found it necessary to recrystallize each sample before determining solubility. This fact, therefore, must be taken into account in interpreting the figures. To obtain a solution of many dyes as strong as the figure given in the table, the dye sample employed must be strictly pure. As this is practically never true, the user of stains can rarely expect to make a saturated solution of the theoretically possible strength. These data nevertheless are useful in indicating the amount of stain to employ in order to obtain a saturated solution.

The data given in Table 7 were compiled in the hope of furnishing information of more practical value to users of stains than those published by Holmes; these were obtained through the courtesy

of the American Pharmaceutical Association. In this table there are listed the solubilities of 50 samples representing 25 commercial certified stains. They are of interest in showing approximately how much deviation to expect between commercial samples of the same dye and about how nearly the solubilities of such samples approach those of the recrystallized dyes listed in Table 6. Frequently the data in Table 7 may prove of more assistance in determining how much dye to employ in making a saturated solution than those given in Table 6.

It would, of course, be still more useful if the solubilities in water and in alcohol of each batch could be printed on the label. Unfortunately, however, no practical method of furnishing this information to the manufacturers and of publishing it on the labels has yet been devised.

INFLUENCE OF IMPURITIES ON INTENSITY OF STAINING

Impurities present in any dye sample or in the solvent not only influence the solubility of the dye but may have a great effect on the intensity of staining. An impurity present may alter the H-ion concentration of the staining fluid; while acid dyes stain better in more acid solutions, and basic dyes in more alkaline solutions. If, moreover, the impurity is a mineral salt or the salt of an organic acid, it may have an effect on intensity of staining even if the reaction of the solution is not altered. Theories to explain these facts are discussed in Chapter III.

The practical lesson from this is that poor staining results with any dye sample are as often to be explained by the presence (or absence) of impurities in the dye, or in the water used as a solvent, as by some irregularity in the dye itself. In many instances the impurities normally present in some dye may be necessary to assure its proper behavior as a stain; and too great effort in the way of purification may be detrimental. This seems to have been unquestionably the case in respect to rose bengal. It has been found (Conn and Holmes, 1928) that a sample of the latter dye, too highly purified for good results if dissolved in distilled water, may stain satisfactorily in tap water containing a certain amount of calcium. It may well happen that the user of some dye which gives too weak staining can increase its intensity of action by adding minute amounts of some mineral salt to the staining solution or by altering the H-ion concentration. In trying this experiment great caution must be employed, for large effects are sometimes produced by minute changes, and it is easy to bring about too weak staining on the one hand, or overstaining on the other if too much of any salt, acid, or base be added.

ASSAY METHODS

When the project of stain standardization was undertaken, in the early '20's, it was realized that reliable methods of assay were needed. They were so definitely lacking at that time that in the early certification work, primary stress was laid on performance of the batches tested; chemical and optical assay methods were at the start more or less incidental. This was regarded as an unsatisfactory and temporary expedient; but it was adopted because tests for performance were then regarded as more reliable than the assay methods then available.

These methods were crude. Dye content had to be determined by means of the rather tricky method of reduction with titanous chloride. This procedure depends on the fact that reducable radicals are present in almost all dyes, so that the dye strength of a sample can be determined by $TiCl_3$ titration, provided that: (1) the end point is sharp; (2) the molecular weight of the dye is known. These provisos make the method sometimes inapplicable. It often happens that the correlation is very poor between titration and colorimetric comparison of various samples of the same dye. As a result considerable research is often needed to establish a reliable assay method; nitrogen determinations or other more absolute procedures must be resorted to in order to establish a satisfactory norm for actual dye content determinations.

In addition to assay for dye content, the spectrophotometer was adopted quite early in the work for determining whether or not any sample was true to type. The original spectrophotometers (see Chapter III) were visual instruments, and it was quite laborious to make accurate readings. The later introduction of photoelectric spectrophotometers has made for greater accuracy, but it has also necessitated some research before standards based on the new type of instrument were well established.

One advantage of the photoelectric instruments is the greater ease in obtaining with them a reading of the density at the peak of adsorption (D_p). This has made it possible to use the spectrophotometer not only for determining the nature of the dye but for comparing the strength of various samples in cases where the titration method has proved inadequate.

The methods at present adopted in the Commission assay laboratory are given in the appendix of this book (pp. 292–328). Constant efforts are being made to improve the methods, and it is realized that they are much better than those adopted in the '20's. Nevertheless they are still unsatisfactory as methods for evaluating the performance of stains; and it is still necessary to continue the practice of testing them in actual use.

HISTOCHEMICAL REAGENTS

The possibility that dyes might some day serve as histochemical reagents was realized quite early. The well known fact that basic

dyes have greater affinity for nuclear substance, acid dyes for cytoplasm is enough to suggest this possibility to anyone; but for a long time the matter remained in the realms of theory, without much concrete fact on which methods could be based.

Undoubtedly the first real stimulus to histochemical technic came from Feulgen and Grossenbeck's application of the Schiff's reagent for aldehyde to cytological structures. (See p. 150). Feulgen was not unaware of what he had accomplished, as shown by the fact that he named the granules thus stained "nucleal" the terminal syllable indicating their supposedly aldehyde-like nature. That was about as far as one could go in those days; not until the various nucleic acids came to be recognized and localized in the cell could the specificity of the Feulgen reaction or the nature of the bodies stained by it be learned.

It was probably ten years or more after Feulgen's work before the reaction named after him became well enough established so that the Stain Commission regarded as a sufficiently important use for basic fuchsin to serve as one of the criteria of certification. It was realized that not all basic fuchsins were suitable for use in the technic; and for some time it seemed an unnecessary refinement to insist that a certified fuchsin should meet this requirement when the dye was so seldom used for the purpose. Now, however, the situation has changed greatly, thanks to the rapid development of histochemical technic, and no basic fuchsin unsatisfactory in the Feulgen procedure would be acceptable.

At present the efforts of histochemists are being directed toward finding other reagents that are specific for the same or other cytochemical features. Some of the reagents are dyes, others are not dyes but develop color in the presence of the chemicals with which they react. The proposal has been made to extend the principle of stain certification to cover some of these reagents. No definite plans to that effect have yet been made, but the project is being given careful consideration at the present time.

CHAPTER III

THE THEORY OF STAINING

THROUGHOUT the other chapters of this book an effort has been made so far as possible to avoid theoretical discussions. Although they contain some statements the truth of which cannot be regarded as fully established, as in the case of the chemical composition of some of the dyes, the discussion in general is confined to observations and to chemical information for which there is good authority, without any attempt to introduce explanations of a theoretical nature. The present chapter, dealing wholly with theory, is included because it is felt that a brief statement of some of the most probable theories to explain staining may be of value in assisting the histologist in the intelligent use of stains for his purposes.

A long theoretical discussion of this subject might be included here, basing it upon the lengthy arguments supporting the various theories that have appeared in the literature. Such a detailed discussion, however, would probably be of little value. Hence this chapter is confined to a bare outline of the important points of the different theories.

Theoretically the dyeing of textile fabrics and the staining of microscopic structures are the same. In one case only the gross effects are observed, in the other the microscopic details. Any theory, therefore, that will explain the details of microscopic staining will be fully adequate to account for dyeing in bulk.

The majority of such theories have depended upon either physical or chemical phenomena rather than on both simultaneously. It would seem at first thought that the dyes combine so firmly with the tissues stained by them that the phenomenon must be a chemical one; but the exponents of physical theories have taken pains to show that all the observed facts can be explained on a physical basis, and that some observations are hard to explain if a chemical union between tissue and dye actually takes place. In a chemical union a new substance is formed which does not necessarily have the properties of either substance entering into its formation, and it is ordinarily impossible to recover the original substances by means of simple solvents. When tissue is stained there is no evidence of any new substance having been formed, the colored tissue merely taking on one of the characteristics of the dye (color) in addition to the properties which it originally possessed; it is, moreover, ordinarily possible to extract all or nearly all of the color by sufficiently long immersion in water, or by the fairly brief action of alcohol. Another observation which points against chemical action is that the tissue never removes the dye completely from solution, even though very dilute; whereas or-

dinary chemical reactions tend to continue until one of the components of the reaction is exhausted. Although these facts are not incompatable with chemical explanations, the original exponents of the physical theories regarded them as full refutation of the possibility of chemical action.

The original dispute concerning physical and chemical theories of stain action is now rather obsolete. Much of it was based on *a priori* argument or on insufficiently understood evidence. Since 1945 there have been various investigations of a more scientific nature, due largely to the growing importance of histochemistry, which are continually bringing out new facts concerning the mechanism of staining. The most recent publications on the subject do not try to differentiate between physical and chemical theories.

The Physical Theory. It was formerly pointed out that all ordinary dyeing or staining phenomena can be explained on a physical basis. There are three factors, all purely physical in nature, which together or separately may explain many, if not all, staining phenomena. In the first place, as nearly all substances stained are more or less porous, simple physical forces such as capillarity and osmosis can account for penetration of the dye into the interior of the tissue. In the second place, the action of *ad*sorption can account for many staining phenomena, even for much of the selective staining with which the biologist is familiar. In the third place, a dye may penetrate some cellular element by *ab*sorption, remaining there in a state of solid solution. Any one or all of these three forces may operate in any given instance; and even the exponents of the physical theory of staining differ in the amount of weight given to each of them. The penetration of the dyes into the tissue by osmotic action is generally admitted; but some students of the subject favor adsorption, others absorption, as the primary explanation of staining phenomena. For a concise discussion of the physical explanations, especially the solution theory, see Holmes (1929 b).

The absorption or solution theory is very simple and the action of some dyes on some kinds of cellular tissue in the presence of mineral salts suggests that this factor is important, at least in some instances. It is also supported by the fact that a dye causes the tissue to become the same color as the dye shows in solution, but not necessarily the same as it shows in its dry form. Dry fuchsin, for example, is green; its solution, however, is red, and so are tissues stained by it, no matter how completely they may be dried. It is nevertheless difficult to explain all staining phenomena, particularly differential staining, on the basis of solution. There are admittedly some special staining phenomena, like fat-staining, where simple solution is the obvious explanation; these, however, form a special case.

Adsorption, on the other hand, furnishes a very satisfactory explanation even of much differential staining, and many once claimed that all staining phenomena might be accounted for in this way. Adsorption is the property possessed by a solid body of attracting to itself minute particles of matter from a surrounding fluid; these particles may be compounds suspended in the fluid, or they may be ions incapable of existing independently except in solution. The principle of selective adsorption is well known to physical chemists, whereby certain ions may be adsorbed by certain substances much more readily than by others. Equally well known is the fact that the rate of adsorption of any ion is strongly influenced by the presence of other ions in the solution, and that an especially profound influence is exerted by the reaction of the solution, in other words by the concentration of free hydrogen or hydroxyl ions in the fluid; the influence of the latter upon the adsorption of basic ions being exactly the opposite from its effect upon the adsorption of acid ions. Such principles as these, well established in physical chemistry, may offer an explanation of such phenomena as the differential staining of different cellular elements, action of mordants, variations in rate of staining with changes in the salt content of the staining solution, and influence of H-ion concentration upon the color assumed by tissue when exposed to the action of both acid and basic dyes. Bayliss (1906) developed this particular phase of the physical theory into what he calls an "electrical theory of staining." See also Parks and Bartlett (1935a and b).

Some of those who held in general to the physical theory of staining admitted that these simple physical phenomena alone cannot explain everything, as for example, instances in which a dye penetrates different cell elements equally readily, but can be easily extracted from some of them while scarcely at all from others. It was assumed, therefore, that the dyes penetrated the cells by mere absorption and diffusion, but were in some cases precipitated there by acids or bases, or other chemical reagents present, thus preventing their extraction by simple solvents. Such a theory admits the possibility of chemical action without assuming an actual chemical union between the dye and the tissue.

This precipitation theory would furnish a satisfactory explanation of the action of mordants. It is well known that certain tissues, that stain feebly if at all with certain dyes, "take" these same dyes deeply if previously treated with the proper chemical. Such a mordant, already present in the tissue, might well cause the precipitation of the dye inside the cell walls. On the other hand the action of mordants may be equally readily explained if it is assumed that staining takes place by means of adsorption or as a process of solution. In the first place, all mordants contain ions that are known to have a decided influence upon the rate of ad-

sorption, and their action may be thus accounted for; they also are nearly always substances that decrease the solubility of any dye and thus increase the readiness with which a dye may enter into solution within the tissue to be stained, if one accepts the solution theory. In other words, the action of mordants can be accounted for by any of the theories of staining.

It is, moreover, frequently pointed out that there is no sharp distinction between chemistry and physics, and in such delicate reactions as those involved in staining, we may be well in the borderland between the two branches of science, where it is impossible to say that a given phenomenon is purely physical or purely chemical. There are, however, certain chemical principles distinctly different from the physical ones just mentioned, that may well enter into the phenomenon of staining; and it is these that were considered most important by the exponents of the chemical theory.

The Chemical Theory. It was claimed on behalf of the chemical theory that just because physical forces alone can explain the facts one is not justified in assuming that chemical unions do not take place when the opportunity for them is present. It is agreed that some parts of the cell are acid in reaction, others alkaline; and it is a well-known chemical principle that the former would tend to combine with the cations in solutions with which they come in contact, the latter with the anions. Now inasmuch as in certain dyes the color exists in the cation (basic dyes) and in others in the anion (acid dyes), it is natural to expect chemical combinations to take place between dye and tissue, depending upon the reaction of the latter. Arguments for the physical theory which exclude chemical action must furnish strong proof that no chemical union occurs; and those who favored the chemical theory claimed that such proof is lacking. That the stained tissue does not present any characteristics to the eye not possessed by either tissue or dye before staining does not prove that no new substance has been formed, nor is this claim refuted by the fact that sufficiently long action of solvents removes the color. Alcohol and even water are not absolutely inert chemically and may withdraw the dye by chemical instead of physical action; the very length of time necessary to remove the color completely (sometimes so long as to allow bacterial decomposition of the tissue) indicates that a rather strong union between dye and tissue has taken place. As to the fact that a tissue which has a strong affinity for some particular dye never withdraws that dye completely from a very dilute solution, those who favored the chemical theory pointed out instances where chemical reactions are known to take place and yet to stop before either component is exhausted. The failure to exhaust the dye completely in a surrounding solution may merely mean that an equilibrium has been reached; chemical

action, moreover, is strongly indicated by the fact that in dilute solutions the tissues take up relatively larger quantities of the dye than in concentrated solution. Accordingly the observation that not all the dye is withdrawn from the solution is a poor argument against chemical action.

Furthermore, if adsorption is assumed to take place only at a specific site (i.e. at the COOH or NH_3 group), the physical and chemical concepts merge into one and cannot be distinguished from each other. It has been shown, for instance, that cells in which the COOH groups are tied up in methyl esters do not stain well with basic dyes. This observation is hard to explain on a purely physical basis; to explain it by adsorption requires the assumption that the phenomenon occurs only at a specific chemical site. Such an explanation comes quite close to assuming chemical action.

In brief, the chemical theory of staining is that the tissues have certain definite chemical affinities which are satisfied by the chemical affinities of the dyes; therefore, when the tissue is put in a solution of the dye the latter combines with those portions of the tissue or of the individual cells which have the proper chemical nature. This theory, it will be seen, is especially well adapted to explain the differential staining which takes place when we find a certain stain acting only on the nuclei. It must be remembered, however, that it is merely a theory. As a matter of fact, the probabilities are that staining is both a chemical and a physical phenomenon.

The chemical theory of staining depended largely upon the question of the acid or the basic character of the dye molecule. It will be recalled that all ordinary dyes are encountered either as sodium or potassium salts of dye acids or as dye salts of colorless acids, the former being the acid dyes and the latter the basic dyes; while certain compound stains are neither acid nor basic dyes, inasmuch as the property of color exists in both the anion and the kation.

The fundamental principle involved in this theory is that certain parts of animal or plant cells are acid in character and hence have an affinity for the basic dyes. The nuclei of the cells, or especially the chromatin within the nuclei, are assumed to be acid in character (due largely to their constituent nucleic acid), and there is no question but that they have a strong affinity for basic dyes; while the cytoplasm has an affinity for acid dyes and is assumed to be basic in character. (See Stearn and Stearn, 1929–30.)

This theory assumes that the acids and bases which go to make up body tissue are ordinarily amphoteric, capable of acting as bases in acid solutions and as acids in basic solutions, the H-ion concentration at which any such compound changes from an acid

to a base in its action being known as its isoelectric point. It also assumes that these compounds, although insoluble, act as though they were electrolytes dissolved in any fluid in which they are immersed. Hence, on this assumption, such a compound acts as a base or as an acid in any staining solution according to whether its isoelectric point is below or above the H-ion concentration of that solution. The chemical theory therefore postulates that any cellular element takes a basic dye if the H-ion concentration of the staining solution is below its isoelectric point, an acid dye if it is above that point.

Now, exactly such a phenomenon as this is to be observed in staining any tissue. With very acid staining solutions even the nuclei take the acid dyes; if one employs successively a series of solutions of decreasing H-ion concentration, the affinity of the nuclei for the acid dyes rapidly becomes less; till at a fairly definite reaction, usually in the region of pH 4, they lose their affinity for acid dyes and take the basic dyes. In solutions near neutrality, therefore, the nuclei take basic dyes, the cytoplasm the acid dyes. Finally at a point considerably to the alkaline side of neutrality even the cytoplasm takes the basic dyes. Such an observation is interpreted to mean that the isoelectric point of the nuclei lies considerably to the acid side of neutrality, that of the cytoplasm considerably to the alkaline side. (This is merely another way of making the statement, given two paragraphs above, that the nuclei are acid in character, the cytoplasm basic.) Now assuming that this interpretation is correct, it might be possible to determine the isoelectric points of different parts of the cell by staining at different reactions. Stearn and Stearn (1928), who were among the leading exponents of the chemical theory in the days of the most heated arguments on the subject, tried to determine in this way the isoelectric points of different species of bacteria. They concluded it to be the pH-value of the crossing point of the staining curves of acid and basic dyes. This idea has been distinctly discredited of recent years. For one thing, staining determinations of any protein must be made on fixed tissue; and the isoelectric point of a fixed protein may well be very different from that of the fresh material. Also, as Singer (1952) points out in his review of the factors which control staining, the position of the crossing point of the staining curves is determined by various other factors, and does not definitely indicate the isoelectric point. He concludes, nevertheless, that although this point cannot be precisely defined by pH staining characteristics, it is possible to compare the curves of different proteins and to learn therefrom the *relative* position of their isoelectric points. Such results show that there is a definite tendency for cationic dyes to unite with anionic elements of the tissue, and *vice versa*.

This is not by any means the whole of the chemical theory of

staining. It is difficult to explain wholly on the basis of various isoelectric points the fact that certain basic dyes have stronger affinities for certain parts of the nuclei than for others, and that of the various cytoplasmic structures outside the nucleus some are more readily stained by certain acid dyes and some by others. In the Flemming triple stain for example, which employs the acid dye orange G and the two basic dyes safranin and gentian violet, with intervening alcoholic differentiation, it is possible to stain the chromatin with gentian violet and the rest of the nucleus with safranin. Before such a phenomenon as this can be explained on a purely chemical basis, it is obvious that we must have a much more detailed knowledge of histo- and cytochemistry than is yet available.

Discussion of the Chemical Theory. The weaknesses of the chemical theory show up particularly in reactions such as this triple stain. Besides the difficulty of accounting for the stronger affinity of certain portions of the nucleus for certain basic dyes and other portions of the nucleus for other basic dyes, there is the difficulty of explaining the action of solvents. The differential staining secured in the Flemming stain, and in fact in the majority of other similar procedures, is brought about not by the staining itself but by the action of solvents which extract some of the dyes more readily from certain portions of the cell than from others. In case a chemical union has taken place between the tissue and the dye, the alcohol or other solvent used must have the effect of breaking down the chemical compound formed between the tissue proteins and the dye molecule, or else it must actually dissolve out the compound in question. It is, however, difficult on the one hand to conceive of alcohol breaking down such a compound; while, on the other hand, if the compound formed is dissolved out of the tissue, it is hard to understand how restaining of decolorized structures is possible.

A further difficulty of the chemical theory arises from the fact that it assumes ionization of the compounds stained, while the tissue is not in solution. Thus, the hypothesis above given assumes a definite isoelectric point for the protein of the tissue, and a physical chemist defines this point as the reaction where the minimum dissociation occurs. Now a protein occurring in a solid form as in sections of fixed tissue can hardly be expected to dissociate, and accordingly, it is a question whether it can properly be said to have an isoelectric point. Under the chemical hypothesis, in short, it is very difficult to conceive how an insoluble solid can behave as an electrolyte and take part in the reactions assumed for it.

One can, however, assume that differential staining indicates differences in chemical nature of the different parts of the cell without adopting the generally accepted chemical theory. Even if the dye is actually taken up by a process of adsorption, this pro-

cess will be greatly influenced by the chemical nature of the different parts of the cell. Substances of an acid character would adsorb basic dyes more readily, and retain them in the adsorbed state more firmly than they would acid dyes, and substances of a basic character would adsorb acid more readily than basic dyes. Amphoteric substances are usually acid or basic in their prevailing character. Acid substances such as cell nuclei exert a decided chemical affinity for basic dyes and accordingly adsorb them readily. They have, however, inferior degrees of affinity for acid dyes and in order to obtain staining with such dyes it is only necessary to convert them into a favorable physical form for adsorption. Basic substances, on the other hand, such as cytoplasm, although ordinarily stained only by acid dyes, may be stained by basic dyes if the latter are employed in a favorable physical form. Favorable conditions for these unusual types of staining are obtained merely by altering the reaction of the staining solutions. Thus one does not have to assume chemical combination between dye and tissue in order to account for the different type of staining obtained in solutions of varying pH value.

It is extremely easy to theorize in regard to the mechanism of staining but very difficult to propose any conclusive argument. Dye chemists have obtained actual quantitative data as to withdrawal of dyes from solution by the fibers of which textiles are made up, which point very strongly to the process being physical rather than chemical. These conclusions are not directly applicable to biological staining for two reasons: In the first place fixed tissues are decidedly different from the animal or vegetable fibers studied by the dye chemists and quantitative data seem to be lacking as to how such tissue removes any dye from solution. In the second place, staining effects are in most instances brought about by the action of decolorizing agents after the staining has taken place, and this introduces a factor which dye chemists have not had to take into account in explaining the affinity of textiles for dyes.

Evidence is still lacking, in fact, to prove or to disprove any of the physical theories on the one hand or the chemical theory on the other. The difference, perhaps, is not one of immense importance. All of the theories teach about the same practical lessons as to the type of dye to select for any particular purpose and as to the influence of salt content or H-ion concentration of staining solution or tissues upon the rate or intensity of staining. The user of biological stains should know enough about the various theories of their action to understand the influence of ionic concentration upon basic dyes on the one hand and acid dyes on the other; but realizing that the same phenomena can be explained by more than one theory, he can safely leave to the physical chemists all discussion of the mechanism involved.

When the first edition of this book appeared, in 1925, frequent papers on the theory of staining were being written by biologists. Today, although much valuable work on the mechanism of staining is being done, it is ordinarily recognized that the findings relate only to specific cases, and arguments about the general theory of staining, as then understood, have almost disappeared from the literature. This is partly perhaps because as we know more we theorize less; but other factors seem to be more important in contributing to the lack of interest. Chief among them is the realization of the great complexity of staining processes, and of the necessity of both chemical and physical theories to explain them—a complexity so great that, until we possess many more facts than at present, our theories are little better than guesses.

A good illustration of the modern way of treating the subject is the recent review paper by Singer (1952). In this paper the author mentions the various theories without showing special favor to any one of them, but gives his attention to the various controllable factors which influence staining of tissues. He points out that the extensive literature of the early part of this century on the theory of staining has brought out many facts of value relating to the interaction of dyes and tissue proteins, and that this interaction deserves further study, without theorizing as to whether it is chemical or physical. His paper is an excellent review of the information on this subject now available.

Histochemistry. It is interesting that now, when a purely chemical theory of staining is not generally accepted, specific instances of what seem to be definite chemical reactions between dyes (or related compounds) and tissue elements are becoming more numerous. The new science of histochemistry depends on the fact that some dyes, or uncolored chemicals which become colored under certain definite conditions, can be used as reagents for giving information as to the chemistry of cell constituents. Although the chemical nature of the observed reactions seems quite evident, such findings have little bearing on the general theory of staining. Ordinary staining follows a comparatively leisurely process of fixation, during which degenerative changes of the tissue can take place; while the histochemical procedure generally involves quick freezing or instantaneous introduction into fixing reagents, or both, to avoid any appreciable change in the nature of the constituents of the tissue. It is clear that the histochemical reagents, therefore, are given quite a different substance to act on from that furnished the ordinary histological stains. Furthermore, the histochemical procedures all employ specific reactions which have little connection with the usual staining methods. An authoritative text on the subject is the book by Gomori (1952).

It is true that, as long ago as the mid-twenties, the chemical theory of staining was leading specialists in the field to speculate

as to the possibility of dyes becoming histochemical reagents (see Conn, 1925), but nothing like the present development of histochemistry was then forseen. Histochemistry, although a fascinating field from the theoretical standpoint, is neither the outgrowth of the general chemical theory of staining, nor does it prove the truth of the latter as a sole explanation of staining.

CHAPTER IV

THE SPECTROPHOTOMETRIC AND CHROMATOGRAPHIC ANALYSIS OF DYES

CHEMICAL methods alone are inadequate in the analysis of many dyes. Not only is the detailed chemistry of some dyes obscure, but the reactions are often complicated by adulterating dyestuffs in such a manner as to preclude entire reliance on rigorous chemical methods. Often a slight change in the arrangement of atoms within the molecule will make a marked change in the nature of the dye, while such a minor change in structure is not always readily detected by chemical means alone. Hence the advisability of employing certain physico-chemical characteristics as displayed by the spectrophotometer in the study of dyes. So important is this study that a discussion of the principles involved is necessary here.

The absorptive spectra obtained by the spectrophotometer are usually characteristic of any particular dye. Quantitative as well as qualitative data may be obtained by the spectrophotometer; and from the combined results nearly all dyes, even though differing from each other only in very minor particulars of chemical structure may be easily differentiated. This method is not only rapid but is also convenient.

The method depends upon the fact that any colored substance absorbs light of certain definite wave lengths and transmits or reflects the rest. The absorption spectrum is essentially the inverse of that which is reflected or transmitted. Therefore the color of light which reaches the eye after transmission through or reflection from a colored substance is complementary to the color of light absorbed by that substance. A violet dye, for example, appears that color because of its predominant absorption of greenish yellow light. The absorption maximum is quite characteristic of any dye; any two dyes having the same absorption curve (a somewhat rare occurrence) are of essentially the same color.

The wave length is ordinarily measured in millimicrons ($m\mu$), or Angstrom units (A) which are $0.1 m\mu$. The visible spectrum begins at around 400 $m\mu$ in the violet and runs to about $750 m\mu$ in the red. The wave lengths of the six most important colors, and their complementary colors are given in Table 1.

A variety of terms and units have come into common usage in connection with the practice of spectrophotometry and numerous symbols to represent them are employed. Since there is some confusion concerning the symbols used, the relationships between the various symbols and units and definitions of the terms most frequently encountered in the literature are given in Table 2.

The color of substances is ordinarily of complex origin, depending

upon the absorption of light in varying degrees, over an extensive spectral range. Whereas the unaided eye is able to register only the composite effect, it is possible to resolve this effect into its component factors with the aid of a spectrophotometer. Although the eye is unable to distinguish between a violet dye and a suitable mixture of a red and a blue dye, the heterogeneous character of the mixture is readily apparent upon spectrophotometric examination. Pure dyes may have simple absorption spectra, in that their light absorption is all at one part of the spectrum, or they may be more complex, showing two or more points on the spectrum at each of which light is absorbed to greater extent than on either side of it. Thus even in the instance of pure products of identical color to the eye, the spectrophotometer frequently reveals decided differences when the character of the light absorption is considered in detail.

TABLE 1

SPECTRUM

(Showing Complementary Colors)

SPECTRUM	WAVE LENGTHS, mμ	COMPLEMENTARY COLORS
Violet	400–430	Yellow
Blue	430–490	
Blue-green	490–510	Orange
		Red
Green	510–570	Purple
Yellow	570–600	Violet
		Blue
Orange	600–620	Blue-green
Red	620–750	

While it may be necessary or advisable in some instances to determine absorption in the infra red or ultra violet, the measurement of absorption within the visible spectrum, upon which color is dependent, is adequate for most purposes. The essential principle of operation of a typical spectrophotometer is best understood by considering first the principles of a visual instrument, although such instruments are now practically obsolete because of the greater efficiency of photoelectric measurements. These principles may be understood by reference to Fig. 1. Such instruments comprise essentially two beams of light from a common source, one to pass through a glass cell containing the sample to be examined, the other to pass through a similar cell containing

solvent only; a photometer for adjusting the relative intensity of the two beams; a spectrometer with prism and eye piece arranged so that portions of the spectra of the light from the two beams are observed juxtaposed in the field of the eye piece. The spectrum of the beam which has passed through the dye solution will be found deficient in those portions which have been absorbed by the dye, and the degree of the deficiency at any position in the spectrum may be measured by determining the degree to which the intensity of the light of the second spectrum must be reduced in

TABLE 2

RELATIONS BETWEEN THE SYMBOLS AND UNITS AND DEFINITIONS OF THE TERMS*

SYMBOLS	DEFINITIONS
A	1. Ångstrom: also Å; 1/6438.4696 of the wave length of the Cd red line, or 10^{-8}cm. = 0.1 mμ. 2. Absorption: 1 − T.
μ	Micron = 10^{-4}cm. = 1000 mμ = 10,000 A.
λ	1. Wave length: expressed in terms of Å or mμ (see 1. above). 2. Wave length of maximum absorption.
I	Transmitted intensity; light transmitted by solute.
I_o	Incident intensity; light transmitted by solvent.
T	1. Transmission, transmittance and transmittancy: I/I_o. 2. Percentage transmission: $100 \times I/I_o$.
E	Extinction: also D or d; $\text{Log}_{10}\ I_o/I$, or $-\text{Log}\ T$.
D	Density: also d or E; (see above)
c	Concentration of solution, gms. per liter.
d	Thickness of solution layer in cm. (Sometimes used to mean density).
k	Specific extinction coefficient: E/cd.
ϵ	Molecular extinction coefficient: Mk, (M = molecular weight).

$I = I_o \cdot 10^{-kcd}$.
$\text{Log}_{10}\ I_o/I = kcd = E$.

*For further discussion of this subject consult Brode (1943), Gibb (1942).

order to obtain an equal intensity in the two fields observed by the eye.

The shutter of the eyepiece may be partially closed so that only a narrow spectral range is visible; this allows the eye to concentrate on the matching of two small fields, each of which appears uniform in color. The instrument is provided with a screw drum, calibrated in wave lengths, by means of which the prism may be rotated in such a manner as to bring light of any desired wave length into the center of the field of vision.

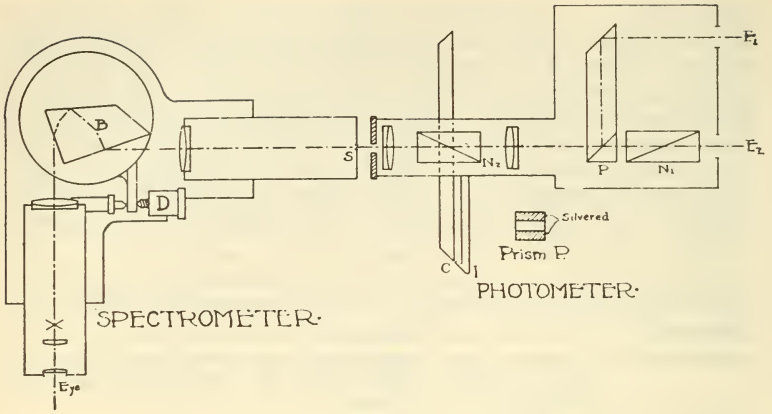


Fig. 1. Schematic diagram of Hilger-Nutting spectrophotometer. Specimen holder with dye dissolved in a suitable solvent is placed at E_1 while a similar cell with solvent alone is placed at E_2. Both are equally illuminated from the same light source. Through the partially silvered prism, P, the upper and lower portion of the photometer field is illuminated through the specimen while the central portion is illuminated through solvent alone. This latter illumination may be varied by the rotation of the analyzer, N_2, which is actuated by a graduated circle C, reading with the index I. By this means the illumination of the several portions of the field is maintained equal and absorption values read off of circle C. The spectrometer of the constant deviation type serves to isolate particular portions of the spectrum by means of a graduated drum D which through a screw actuates the prism table supporting prism B.

This visual type of instrument, as explained above, is now rarely used, having become obsolete on account of the greater efficiency of the photoelectric cell in comparison with the eye. The eye is badly subject to fatigue after continuous use, and even at its best has low sensitivity at the violet end of the spectrum. The photoelectric cell, on the other hand, is not so subject to fatigue, can make readings over a longer wavelength range, and even into the ultra-violet, if special equipment for that purpose is provided. For these reasons more rapid readings can be made, and it is possible to obtain the exact point of maximum absorption much more quickly than with the eye. A photoelectric instrument such as the General Electric Recording Spectrophotometer, is even more automatic, as it has a motordriven device that records even the most complicated absorption curve on a revolving drum in only a few minutes. A recording spectrophotometer, however, is a rather unnecessary refinement in the case of dyes, because of their relatively simple absorption curves. Simple non-recording photoelectric instruments, such as the Beckman or the Coleman, are ordinarily sufficient.

The optical principles of the Beckman are given in Fig. 2 and a photograph of an assembled instrument in Fig. 3. Details of

the Coleman instrument are not given; in principle it is quite similar. In theory, such an instrument merely replaces the eye with a photoelectric cell; but in actual practice the optical system is different. This is due to the fact that the photoelectric cell enables one to measure directly the intensity of the light by means

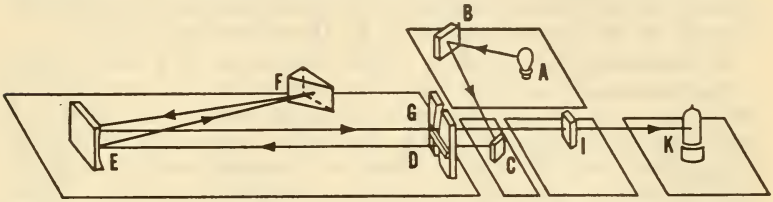


FIG. 2. Schematic optical diagram of Beckman model DU spectrophotometer

A. Light source
B. Condensing mirror
C. Diagonal mirror
D. Entrance slit
E. Collimating mirror
F. Quartz prism
G. Exit slit
I. Absorption cell
K. Photoelectric tube

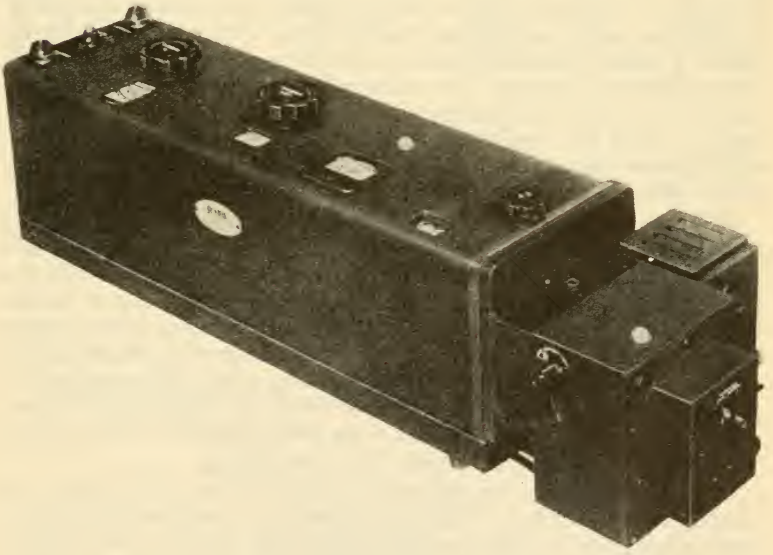


FIG. 3. Photograph of an assembled Beckman model DU spectrophotometer

of a potentiometer. With the eye as the recording agent, two beams of light are necessary, one passing through the unknown, the other through the solvent alone, plus an analyzer with which the intensity of the light can be varied until the two beams of light are equal in apparent strength. With the photoelectric cell, on

the other hand, simultaneous inspection of two beams of light is not necessary, nor is the analyzer for reducing the light intensity needed; hence a single beam of light passing through the instrument is all that is needed, with resulting greater simplicity of the optical system. The chief bulk of a non-recording photoelectric spectrophotometer is occupied by a potentiometer to record the strength of the current generated by the photoelectric cell.

In measuring the complete visible absorption of a dye, a series of measurements is made over the portion of the spectrum in which any appreciable absorption may be noted. In doing this with such an instrument as the Beckman, one of the absorption cells is filled with the solvent alone, as a control, another with the dye dissolved in the same solvent, in quite dilute solution. Then, after checking the instrument as to its zero point the control cell is placed in the beam of light coming from the exit slit, the monochromator set to a point somewhat to one side of that part of the spectrum where absorption is expected, and a reading taken as to the intensity of the light reaching the phototube. Then a similar reading is taken with the unknown sample. The setting is then changed to a slightly different wavelength, and new readings taken. In practice such readings are taken about every 5 mμ in the regions of slight absorption but at least every 1 mμ near the peak. The difference between the readings of the control and the unknown at any point indicates the absorption of light at that point. With the Beckman, four absorption tubes are provided, which allows for readings to be obtained one after the other with three different unknowns.

If measurements are carried out under suitable standardized conditions, the spectral position and the general form of the absorption curve are characteristic of the individual dye, while the magnitude of absorptive indices (the height of the curve) varies directly with the amount of dye present. The absorption curves of dyes which are very closely related in structure are sometimes so similar as to be practically identical. In such instances the individual dyes may be recognized by means of quantitative determinations of the degree in which their absorption is modified under the influence of suitable variations in conditions.

The absorption curves of typical yellow, orange, red, violet, and blue dyes are recorded in Figure 4. It will be noted that their maximum absorption in each case falls within the range of the complementary color (cf. Table 1). The great majority of dyes of these colors, in the usual solvents and under the usual conditions, show but one absorption band in the visible spectrum. The curves are seldom perfectly symmetrical, however, and usually give indications of localized secondary absorption in some portion of the band. It has been shown that this secondary absorption is due, in numerous instances, to a tautomeric form of the dye. It should never be

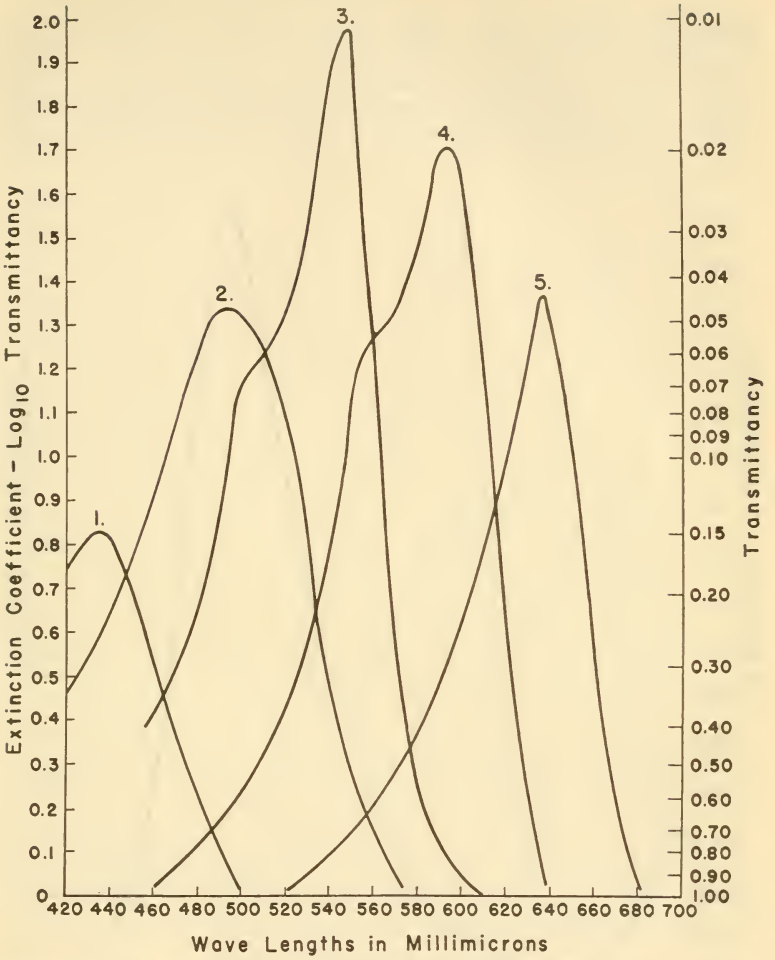


FIG. 4. Absorption curves of five dyes of different colors:

1. Tartrazine (yellow)
2. Orange G.
3. Fuchsin (red)
4. Crystal violet
5. Neptune blue BG.

accepted as evidence of the presence of a second dye unless it has been ascertained that it is not found with a pure sample of the dye under conditions of examination.

The absorption curve of a green dye is recorded in Fig. 5. It has

FIG. 5. Absorption curve of malachite green.

a principal band in the orange and a secondary band in the violet. Both the absorption curve and the color of the dye could be matched closely by mixing a suitable blue and a yellow dye in the correct proportions. All green dyes absorb appreciable amounts of violet light as well as of red light.

In Fig. 6 is given the absorption curve of a dye mixture, together with the curves of the component dyes. The mixture is reported to have been marketed in good faith as asymmetrical dimethyl thionin, a dye which is intermediate in constitution and in color between thionin and methylene blue (see under azure A, on p. 109). The absorption curve plainly indicates the presence of two dyes,

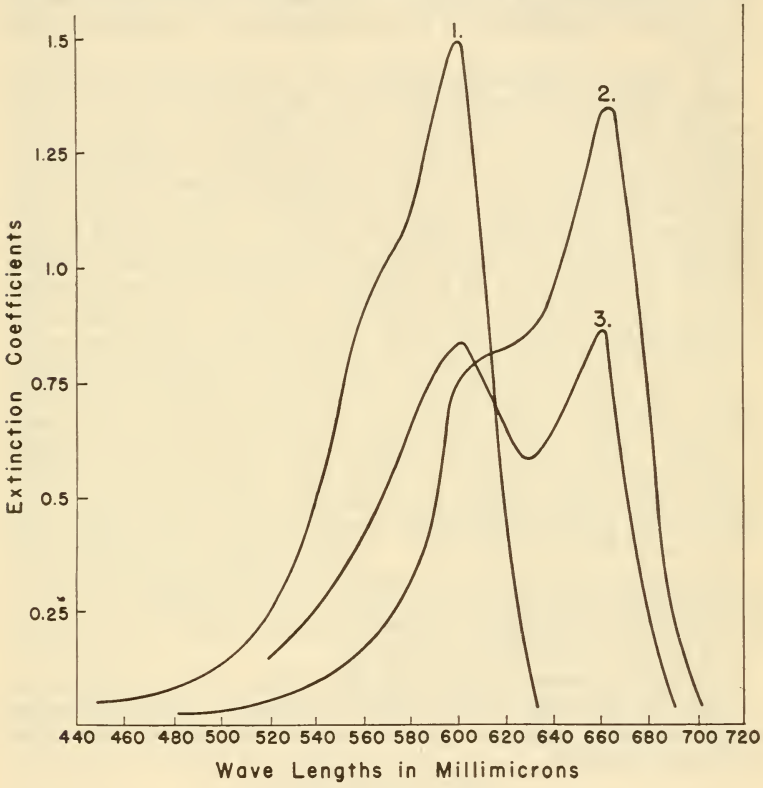


Fig. 6. Absorption curves of:

1. Thionin.
2. Methylene blue.
3. Apparent mixture of these two dyes, incorrectly labelled (although in good faith) a dye intermediate between them in chemical composition.

and suggests their probable identity. (It would be advisable to effect the separation of small amounts of both dyes, if their positive identification is desired.) The color of the mixture is very similar to that of dimethyl thionin. The absorption curve of that dye, however, is a simple and well-defined curve resembling those

of thionin and methylene blue, but occupying an intermediate position in the spectrum.

This illustration shows how valuable the spectrophotometric analysis may be in determining whether a given product is a simple dye or a mixture of two or more dyes. This fact, together with its use in determining the exact shade of any dye, makes it the most valuable test to apply to a stain, other than to determine by actual use whether the sample will prove satisfactory to the microscopist or not.

The user of biological stains may well question, however, whether spectrophotometric data have any actual value for him in guiding him in the use of dyes. Such data as are given in this book do in fact have a distinct use, provided he understands the principle underlying them. Let us suppose, for example, that never having heard of pyronin, he did not know its color; in such a case the absorption maximum (552) as given on p. 178 would furnish him this information. By referring to the spectrum and its complimentary colors on page 54 he would find that an absorption maximum at 552 means a primary absorption of green light; and as the complementary color is red, he would thus learn that pyronin is a red dye. In a similar way if he were trying to decide between using one or the other of two closely related dyes, knowledge of their absorption maxima might help him, provided the decision depended on shade. Thus if he were using azure A and obtained a shade somewhat too red, the spectrophotometric data given on pp. 107–110 would show him whether to substitute azure B or azure C. It will be seen that the absorption maxima of azures A, B, and C are respectively 628, 652, and 609. Reference again to the Table 1 on p. 54 shows that the complementary color corresponding to wavelength 652 would be on the blue side of 628, while 609 would be on the red side; accordingly azure B should be selected if bluer staining than with azure A were desired.

It should be remarked that the absorption curve of a dye is not necessarily the same in two different solvents. In the following pages aqueous solutions are to be understood unless some other solvent is specified.

CHROMATOGRAPHY

Recently more and more stress is coming to be laid on a different method of dye analysis known as chromatography. It has no real relation to spectrophotometry, but it is considered in this chapter because it also furnishes a means of separation and identification of dyes on the basis of differences in color that are not shown by simple visual comparison.

When two different chemicals are in solution together and are brought into contact with porous substance in which capillary rise readily takes place, it is rare that both compounds will pass through the pores of the substance at the same rate. The result is the

production of a column in which each compound tends to be located in definite bands. This phenomenon was naturally first observed in the case of colored compounds; hence the name chromatography, although it is now used, fully as much for uncolored as for colored compounds. Dyes lend themselves naturally to this method of analysis; although until recently it has not been much used for them. The method is of value not only for showing the various bands of color that develop, but actually to isolate the individual constituents of the mixture, as the column can be cut into its various sections and the chemical there localized can be eluted by the proper reagent and thus obtained in pure form.

The method as originally developed called for a column of alumina in a glass tube, in which the bands are located at points determined by the differing degrees of adsorption taking place between the alumina and the different constituents of the mixture. A more recent development, known as paper partition chromatography, depends on a different principle, the relative solubility of different compounds in two solvents, together with the affinity of cellulose fibers for the solvents, which determines the rate of passage through the pores of the filter paper. The paper partition method has several advantages, among them the ease with which the chromatograms can be preserved and with which sections can be removed for extraction of the individual fractions. Simultaneous papers (Emery and Stotz, 1952; Christman and Trubey, 1952) have described the application of the technic to dye analysis; and at about the same time, Evans and Walls (1952) have shown the possibility of getting very similar chromatographic pictures by the use of filter paper electrophoresis.

A chromatographic strip obtained in this way from a mixture of dyes can be very striking. Emery and Stotz show that a mixture of three dyes (eosin Y, orange G, and erythrosin B) in a mixture of butanol and water, and allowed to diffuse from a spot at the top of a filter paper strip, develops five zones of color, one orange, one red, two pink and fluorescent, one fluorescent without color in ordinary illumination; of these five, the three fluorescent zones all come from eosin Y, the other two from orange G and erythrosin respectively. These authors, by cutting out the various zones have been able to isolate the individual dyes and to figure the concentration of each in the original mixture.

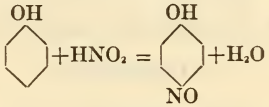
This illustration is given merely to show the probable value of the method. Much more work must be done, before it can be used in routine dye analysis, and it is not likely to replace spectrophotometry; but, as shown by Emery and Stotz it can serve to reveal the identity of dyes in a mixture, even though one of them (in this case eosin Y) is present in such strength as to mask the others when analysis must depend on the spectrophotometer. Accordingly it is felt that chromatography may have an important place in future assay methods for biological stains.

CHAPTER V

NITRO AND AZO DYES

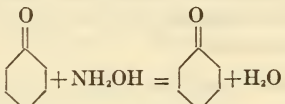
1. NITROSO DYES (QUINONE OXIMES)

The nitroso dyes are produced by the action of nitrous acid on phenolic compounds, according to the equation



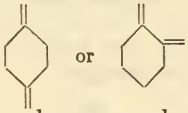
(*nitrosophenol*)

The fact, however, that the quinone oxime produced by the action of hydroxylamine on p-quinone according to the equation



(*quinoneoxime*)

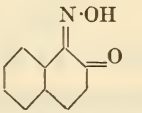
is identical with the nitrosophenol produced as shown above, shows that in all probability the substances can react as quinone-oximes, which better accounts for their behavior as dyeing agents since the quinone ring



occurs frequently in other dyes as a chromophore (see page 30).

A3 NAPHTHOL GREEN Y C. I. NO. 2*

Synonyms: *Fast printing green. Gambine.*



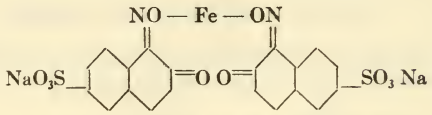
$C_{10}H_7NO_2$; *Mol. Wt.* 173.164

(*An acid dye*)

*This abbreviation stands for the number in the "Colour Index;" see Chapter III, p. 36.

A8 NAPHTHOL GREEN B C. I. NO. 5

Synonyms: *Naphthol green. Green PL. Acid green O.*
This dye probably has the formula:



$C_{20}H_{10}N_2O_{10}S_2Na_2Fe$; *Mol. Wt.* 604.260

(*An acid dye*)

Some naphthol green, probably the second of the above two dyes, has recently been employed by Volkmann and Strauss (1934) as a component of a tissue stain, the other dyes used in combination with it being gentian violet and azocarmine G. Mollier (1938) calls for it in a quadruple tissue stain, with orcein, iron-hematoxylin, and azocarmine G.

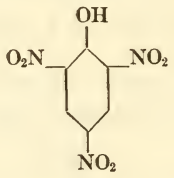
For the technic of **Mollier's quadruple stain,** see Staining Procedures*, p. IB_3-7.

2. NITRO DYES

In this group the chromophore is $-NO_2$. The chromophore is of such a strongly acid character that the dyes of this group are all acid dyes.

B5 PICRIC ACID C. I. NO. 7

Picric acid is formed by the action of nitric acid on phenol, thus introducing three nitro groups:



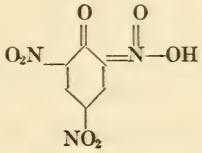
$C_6H_3N_3O_7$; *Mol. Wt.* 229.108

(*An acid dye; absorption maximum about 360 in alcohol*)†
Solubility at 26°C: in water 1.18%; in alcohol 8.96%

*Conn and Darrow (1943-4).
†When not otherwise specified, the absorption maximum is given for an aqueous solution.

This compound forms salts by the dissociation of the –OH group, and the salts have considerable value as stains. Ammonium picrate is most commonly thus used.

According to Dehn and Ball (1917), picric acid also has an isomeric form



This ortho-quinoid form occurs simultaneously with the benzoid form, and changes in color of the compound may be explained by disturbance of the equilibrium between the two isomers. The quinoid isomer, with its -NOOH group, might well account for the strongly acid character of the compound.

Picric acid (or one of its salts) is quite extensively employed in contrast to acid fuchsin in the Van Gieson (1889) connective tissue stain (which was originally proposed for staining nervous tissue). It is also used as a general cytoplasmic stain in contrast to the basic dyes. Wolf (1938) recommends it as a counterstain to crystal violet in the cytology of fungi; Astbury and Preston (1940) for staining cell walls of marine algae. It has further application as a fixative for tissues that are to be sectioned and as a slow decalcifying agent.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED*

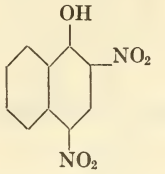
NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Van Gieson's stain with iron hematoxylin	IA_4-21
Picro anilin blue with Biebrich scarlet	IB_3-12
Modified Gallego elastic tissue stain	IB_3-15
Schmorl's method for bone sections	ID_3-12
Picro anilin blue with safranin for woody sections	IIA-11
Goodpasture's stain for influenza bacilli in tissues	$IIIB_3$-15
MacCallum's stain for influenza bacilli in tissues	$IIIB_3$-14

*Under this heading are given references to procedures described in detail in Staining Procedures, edited by Conn and Darrow (1943-4).

B10 MARTIUS YELLOW C. I. NO. 9

Synonyms: *Manchester yellow. Naphthol yellow.*

This dye is usually the sodium, or sometimes calcium or ammonium, salt of the following acid:



$C_{10}H_6N_2O_5$; *Mol. Wt.* 234.164

(*An acid dye; absorption maxima about 445, [399, 379]*)
Solubility of sodium salt at 26°C: in water 4.57%; in alcohol 0.16%. Solubility of calcium salt: in water 0.05%; in alcohol 1.90%

Martius yellow has been used by Pianese (1896) in combination with malachite green and acid fuchsin for studying cancer tissue; the same technic was applied to plant tissue by Müller (1912), and is now quite extensively used by plant pathologists in studying sections of tissue infected by fungi. More recently it has been employed by Nebel (1931) in contrast to resorcin blue for staining pollen tubes in styles. Halbert (1935), by adding it to bacteriological media, finds that it favors the growth of *Escherichia coli*, but inhibits *Aerobacter aerogenes*. The dye is also used in preparing certain light filters used in photomicrography.

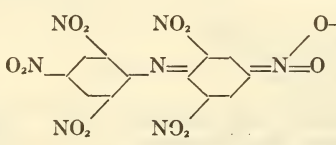
This dye is not at present available in America.

For technic of **Pianese III B Stain**, see Staining Procedures, p. IIA-12.
For technic of **Lacmoid-martius-yellow stain for pollen tubes**, see Id. p. IIA-14.

B15 AURANTIA C. I. NO. 12

Synonym: *Imperial yellow.*

This dye is the ammonium salt of hexanitro-diphenylamine.



$C_{12}H_8N_8O_{12}$; *Mol. Wt.* 456.248

(*An acid dye; absorption maximum about 425*)

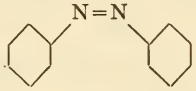
Solubility at 26°C: in water nil; in alcohol 0.33%

It is obsolete as a textile dye, although sometimes used as a photographic desensitizer. It is called for in combination with toluidine blue and acid fuchsin in the Champy-Kull technic for demonstrating certain cell constituents (mitochondria, etc.) It is very poisonous, frequently causing severe dermatitis.

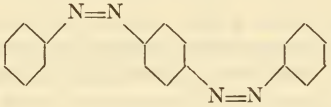
Kassanis (1939) employs it in the Kull technic (i.e. with acid fuchsin and toluidine blue) for the study of virus infested plants. Favorsky (1939) uses it for inducing polyploidy in plants.

3. THE AZO GROUP

The azo dyes are characterized by the chromophore —N=N— joining benzene or naphthalene rings, thus:

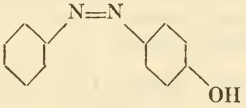


It is possible for the azo group to occur more than once in a molecule, forming the disazo dyes, thus:

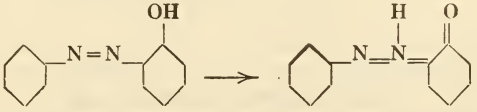


The azo chromophore is distinctly basic; but not sufficiently so to make the dyes basic when they contain hydroxyl groups. Those containing amino groups are, of course, pronouncedly basic.

The position of the hydroxyl or amino group on a benzene ring in relation to the azo group is important. Ordinarily they are in the para position to each other, thus:

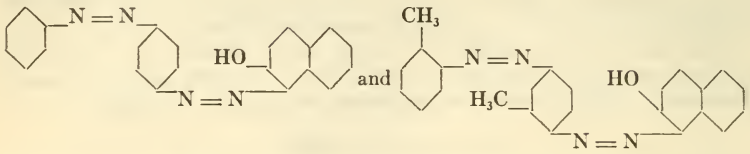


The ortho position is next frequently assumed; rarely the meta position. When the hydroxyl group assumes the ortho position the character of the compound is quite distinct from that of the para compounds. By a rearrangement of the atoms such a compound is sure to change to a quinoid form, thus:



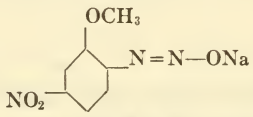
A compound of this latter structure cannot form salts and does not act as an ordinary dye. It does, however, prove to be soluble in

oil and is able to color it by an apparently physical process. Hence the azo-ortho-phenols, or azo-beta-naphthols, like Sudan III and Sudon IV,



are important fat staining dyes.

B25 NUCLEAR FAST RED SALT R



$C_7H_4N_2O_4Na$; *Mol. Wt.* 217.123

This compound is not a dye, but is mentioned here because it forms an azo dye when combined with phenols and certain phenolic derivatives, and is used as a histochemical reagent, producing a bright red in the tissue in the presence of such compounds. It is best known to the biologist by the German name "Kernechtrotsalz B" under which it has been sold by a stain company in that country. Clara (1935) describes the use of such compounds for this purpose. Jacobson (1939) similarly employs it in the study of argentaffine cells.

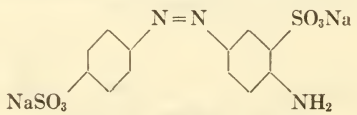
The compound is really a diazonium salt like those described in Chapter X among the histochemical reagents. It is a diazo salt of 5-nitro-2-amino methoxybenzene closely related to National Aniline's fast red salt B (see p. 234).

a. Mono-Azo Dyes

C1 FAST YELLOW C. I. NO. 16

(*Echt Gelb*)

Synonyms: *Fast yellow FY, G, S, BG, etc. Acid yellow.*



$C_{12}H_9N_3O_6S_2Na_2$; *Mol. Wt.* 401.330

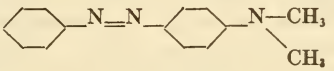
(*An acid dye; absorption maximum about 490 in acid solution*)

Solubility at 26°C: in water 18.40%; in alcohol 0.24%.

This dye is rarely used as a biological stain, but is called for by Schaffer for staining sections of bone, and by Unna in certain stain mixtures used in studying the phenomenon called by him chromolysis. Fast yellow G or GG is employed by Wallart and Houette (1934), together with hematoxylin and acid fuchsin, in a trichrome staining technic.

c5 OIL YELLOW II C. I. NO. 19

Synonyms: *Butter yellow. Oil yellow D. Fast oil yellow B.*



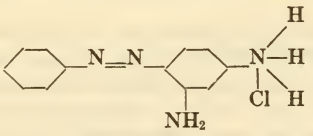
$C_{14}H_{15}N_3$; *Mol. Wt.* 225.284

Butter yellow is the most common designation for this dye in the biological literature. The name is unsatisfactory for two reasons; first, it sometimes falsely suggests some relationship to butter; second, it is ambiguous because C. I. No. 17 (amino-azo-toluene) is also called butter yellow. For these reasons the name given in the above heading is preferred; or one may definitely specify it by the chemical term, *p*-dimethyl-amino-azo-benzene.

It has not been used, apparently, as a stain, but has been considerably studied as a carcinogenic substance, so much so that fear has been expressed in some quarters that the public might come to associate cancer with butter and that this food might be thereby discredited.

c8 CHRYSOIDIN Y C. I. NO. 20

Synonyms: *Brown salt R. Dark brown salt R.*



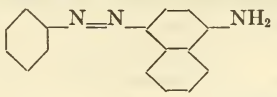
$C_{12}H_{13}N_4Cl$; *Mol. Wt.* 248.713

(A basic dye; absorption maximum about 461)

Solubility at 26°C: in water 0.86%; in alcohol 2.21%

This dye is a good substitute, in some procedures, for Bismarck brown. Like the latter, it is easily injured by heat, and boiling should be avoided in preparing solutions. Maheshwari and Wulff (1937) have employed it in the vital staining of pollen tubes; Lison (1938) for the vital staining of insects. Monné (1939) uses it to demonstrate the Golgi apparatus. Varco and Visscher (1941) include it among a series of dyes employed in the study of gastric secretion.

c11 BENZENE-AZO-*α*-NAPHTHYLAMINE



$C_{16}H_{13}N_3$; *Mol. Wt.* 247.288

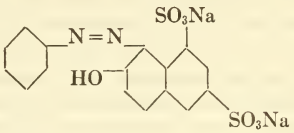
(*Absorption maximum of hydrochloride 557.6 in alcohol*)

A colored compound recently employed by Carter (1933) as a vital stain for protozoa (probably in the form of its hydrochloride) and given the abbreviated name "BAN". This product has apparently never been put on the market as a commercial dye; only its formula and chemical properties are known.

c13 ORANGE G C. I. NO. 27

Synonym: *Wool orange 2G.*

Slightly different grade: *Orange GG, GMP. Crystal orange GG.*



$C_{16}H_{10}N_2O_7S_2Na_2$; *Mol. Wt.* 452.370

(*An acid dye; absorption maximum about 476–480*)

Solubility at 26°C: in water 10.86%; in alcohol 0.22%

This dye is strongly acid because of the two sulfonic groups. It is one of the most valuable plasma stains in histological work. It has great use as a background stain for hematoxylin and other nuclear dyes in cytology. It is frequently employed, both by botanists and zoologists, as a cytoplasmic stain, together with the two nuclear dyes safranin and gentian violet in the Flemming triple stain. It is of importance to the pathologist for its use with anilin blue and acid fuchsin in the Mallory connective tissue stain; and is used in various other double and triple staining methods, such as that of Ehrlich-Biondi-Heidenhain, in which it is mixed with methyl green and acid fuchsin. The Ehrlich "triacid mixture," also a combination of these same three dyes, is used in staining blood. A further use is Bensley's "neutral gentian," a combination of orange G and gentian violet for staining the A and B cells in the islands of Langerhans. An important recent application is by Stoughton (1930), with thionin, for the differentiation of parasites in plant tissue.

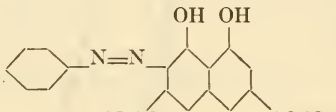
A spectrophotometric graph of this dye, compared with two other orange dyes, is given in Fig. 7, page 74.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Mallory's anilin blue collagen stain	IB_3-3
Heidenhain's "Azan" modification	IB_3-4
Crossman's modification	IB_3-5
Acid alizarin blue modification	IB_3-6
Kornhauser's "Quad" stain	IB_3-8
Stoughton's thionin and orange G for fungi in tissue	IIA-12
Flemming's triple stain	IIB-10
Id., with iodine	IIB-11
Feulgen stain, De Tomasi modification	IIB-13

c16 CHROMOTROPE 2R C. I. NO. 29

Synonyms: *Chromotrope N2R. Chromotrope blue 2R. Fast fuchsin G. XL Carmoisine 6R. Acid phloxine GR.*



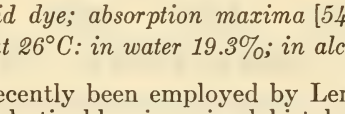
$C_{16}H_{10}N_2O_8S_2Na_2$; *Mol. Wt.* 468.370

(An acid dye; absorption maxima [542] 503)
Solubility at 26°C: in water 19.3%; in alcohol 0.17%

This dye has recently been employed by Lendrum (1935) as a counterstain to celestin blue in animal histology. Lillie (1940) finds it is a fairly good substitute for "xylidine ponceau" in the Masson (1929) trichrome stain. Crossmon (1940) uses it in the selective staining of red blood cells in sections.

c19 AZOPHLOXINE GA C. I. NO. 31

Synonyms: *Fast crimson GR. Amidonaphthol red G.*



$C_{18}H_{13}N_3O_8S_2Na_2$; *Mol. Wt.* 509.422

(An acid dye; absorption maxima 540, 502)

Very little mention has been made of this dye in the biological literature but it has been called for by Dublin (1943) with "ponceau de xylidine" as one step of the Bodian stain in neuropathology.

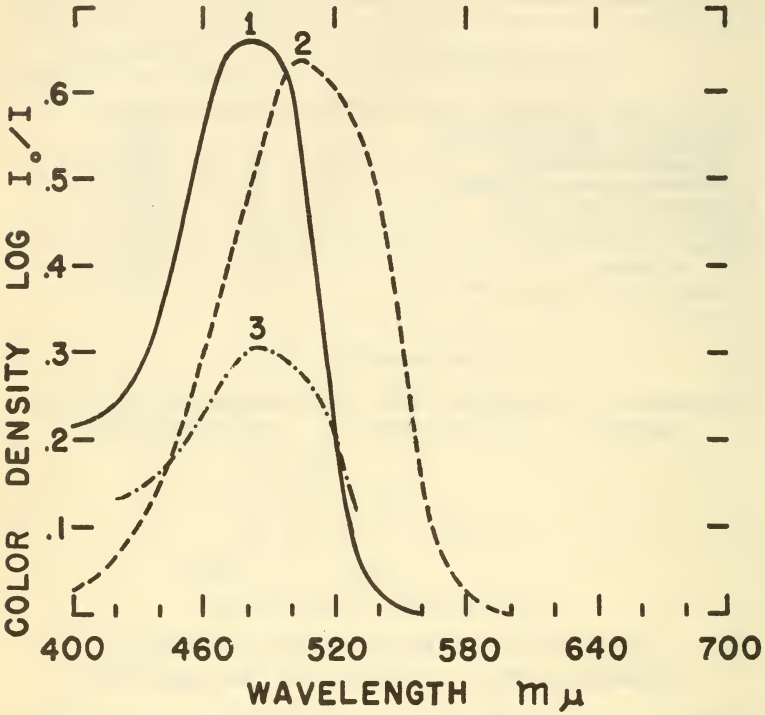
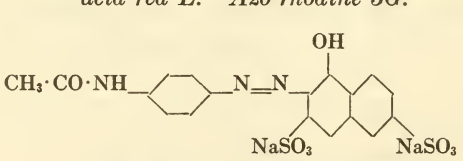


FIG. 7. Spectral curves of three azo dyes:
1. Orange G. 2. Methyl orange. 3. Orange II.

c21 SORBINE RED C. I. NO. 54

Synonyms: *Azofuchsin 3B. Kiton red S. Eriorubine G. Azo acid red L. Azo rhodine 3G.*



$C_{18}H_{13}N_3O_8S_2Na_2$; *Mol. Wt.* 509.422

(*An acid dye: absorption maxima about [545], 499*)

Lillie (1940) suggests this dye (under the name Azofuchsin 3B) among possible substitutes for "ponceau de xylidene" in the Masson trichrome technic.

AZO FUCHSIN

There is an occasional reference in histology to the use of an azo fuchsin. Such references are not sufficiently definite, however, as there are several different azo fuchsins. The following, for example, are recognized in the Colour Index:

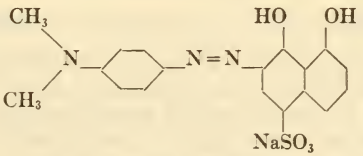
Aso fuchsin 4G	C. I. No. 29
Azo fuchsin	C. I. No. 30
Azo fuchsin 3B	C. I. No. 54 (see p. 74)
Azo fuchsin 6B	C. I. No. 57
Azo fuchsin B	C. I. No. 66
Azo fuchsin G	C. I. No. 153
Azo fuchsin 6B, GN or S	C. I. No. 154

All of these acid mono-azo dyes are closely related chemically to orange G and Bordeaux red.

Of these, Lillie (1948) definitely specifies C. I. No. 153 as a constituent of his naphthol blue black tissue stain.

c25 AZO ACID BLUE B C. I. NO. 59

There are various azo acid blues, with various shade designations attached to the names, but whose synonymy is somewhat confusing. They are mixtures; but an important constituent is regarded as being



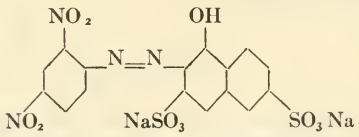
$C_{18}H_{16}N_3O_5SNa$; *Mol. Wt.* 409.389

(*An acid dye*)

Menner (1935) has called for this dye in a stain for nervous tissue. Otherwise there seem to be few, if any, references to it in the biological literature.

c28 NITRAZINE

Synonyms: *Nitrazine yellow. Delta dye indicator.*



$C_{16}H_8N_4O_{11}S_2Na_2$; *Mol. Wt.* 542.370

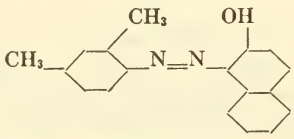
(*An acid dye*)

This dye has recently been proposed as an indicator, having a pH range of 6.0 to 7.2. Its acid color is bright yellow, from which it passes thru gray-green into its alkaline color, bright blue.This indicator can be used in the form of test strips, like litmus paper. It is more sensitive than litmus, however, and shows greater color contrast. Lillie (1940) finds it can replace xylidine ponceau in the Masson (1929) trichrome stain.

For technic of **Masson trichrome stain, Lillie modification,** see Staining Procedures, p. $1B_3$-18.

c31 SUDAN II C. I. NO. 73

Synonyms: *Oil scarlet. Fast oil orange II. Red B. Fat ponceau. Orange RR.*



$C_{18}H_{16}N_2O$; *Mol. Wt.* 276.324

(*An acid dye; absorption maxima in alcohol: 531.5, 494.5*)

Solubility at 26°C: in water nil; in alcohol 0.39%

In this dye the hydroxyl group is in the ortho position with respect to the azo group. As explained above (p. 69), such compounds show a tendency toward intramolecular rearrangement so that the hydrogen atom detaches itself from the hydroxyl group and becomes fixed to the neighboring nitrogen. Such a compound is neither acid nor basic and, not being able to form salts, is not an ordinary dye, but is fat soluble and has the power of coloring fat. Therefore, this compound, together with the following and several others of similar structure described below, are fat stains.

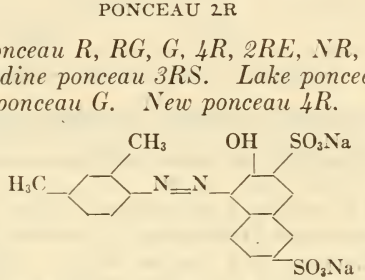
This fat stain has frequently been confused with oil red O because both are mistakenly given in the Colour Index as synonyms.

Lillie (1944c) has tried both dyes in his supersaturated isopropanol technic, and finds Sudan II satisfactory, better in fact than Sudan III, although not so intense or yielding such a stable solution as oil red O, oil red 4B or Sudan red 4B, it is especially good as a fat stain for central nervous tissue, in combination with Weigert's technic for myelin. (See p. 88.)

For **Lillie's supersaturated isopropanol technic**, see Staining Procedures, p. ID_3-20.

c35 PONCEAU 2R C. I. NO. 79

Synonyms: *Ponceau R, RG, G, 4R, 2RE, NR, J, FR, GR. Scarlet R. Xylidine ponceau 3RS. Lake ponceau. Brilliant ponceau G. New ponceau 4R.*



$C_{18}H_{14}N_2O_7S_2Na_2$; *Mol. Wt.* 480.422

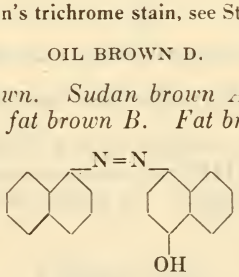
(*An acid dye; absorption maxima about* [*538*] *499*)

A dye called xylidine ponceau, possibly this one, has been used as an histological counterstain in the Masson technic (see Foot, 1933), and by others who have followed Masson's methods with certain modifications. Lillie (1940), however, suggests other dyes for this purpose which give better results. The exact identity of the French "ponceau de xylidine" employed by Masson is not known.

For the technic of **Masson's trichrome stain**, see Staining Procedures, p. $1B_3$-18.

c38 OIL BROWN D. C. I. NO. 81

Synonyms: *Sudan brown. Sudan brown AN. Fast oil brown* S. *Brilliant fat brown B. Fat brown III.*



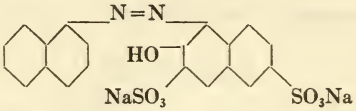
$C_{20}H_{14}N_2O$; *Mol. Wt.* 298.328

Lillie (1944c) recommends Sudan brown, Sudan brown 5B, and oil brown D as satisfactory fat stains in the supersaturated isopropanol technic. These dyes all undoubtedly represent shades of the above.

c41 BORDEAUX RED C. I. NO. 88

Synonyms: *Fast red B, BN or P. Cerasin R. Archelline 2B. Azo-Bordeaux. Acid Bordeaux.*

Various grades denoted: *Bordeaux B, BL, G, R, R extra.*



$C_{20}H_{12}N_2O_7S_2Na_2$; *Mol. Wt.* 502.426

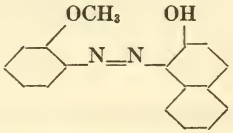
(*An acid dye; absorption maximum about 520*)

Solubility at 26°C: in water 3.83%; in alcohol 0.19%

Bordeaux red is used as a cytoplasmic stain, as, for example, in aqueous solution when Heidenhain's hematoxylin is to be used immediately afterward as a nuclear stain. It has also been used with thionin and methyl green for staining sections, particularly of spleen, testis, and liver. Lillie (1940) finds it a fairly satisfactory substitute for xylidine ponceau in the Masson technic.

c45 SUDAN R C. I. NO. 113

Synonyms: *Brilliant fat scarlet B. Oil vermillion.*



$C_{17}H_{14}N_2O_2$; *Mol. Wt.* 278.298

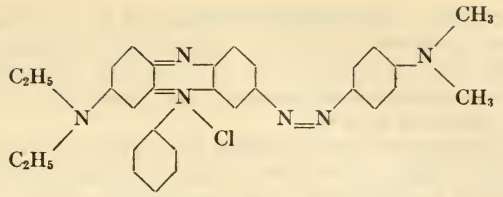
(*A weakly acid dye; absorption maxima 569 [519.8]*)

Weichherz (1934) has recently used a Sudan R III (possibly this dye or possibly Sudan III) in connection with the Kahn reaction for the diagnosis of syphilis. It makes the reading of the tubes easier.

c50 JANUS GREEN B C. I. NO. 133

Synonyms: *Diazin green S. Union green B.*

This is an azo dye having an azin as well as an azo chromophore group, and is thus related to the safranins. It is a compound of diethyl safranin with dimethyl anilin through an azo group.



$C_{30}H_{31}N_6Cl$; *Mol. Wt.* 511.053

(*A basic dye; absorption maximum about 610–623*)

Solubility at 26°C: in water 5.18%; in alcohol 1.12%

Janus green is best known for its use in demonstrating chondriosomes, stained *intrà vitam*, according to the technic of Michaelis (1900), and as more recently developed by Cowdry, and by Bensley (1911). It is also used by Faris (1924) with neutral red for

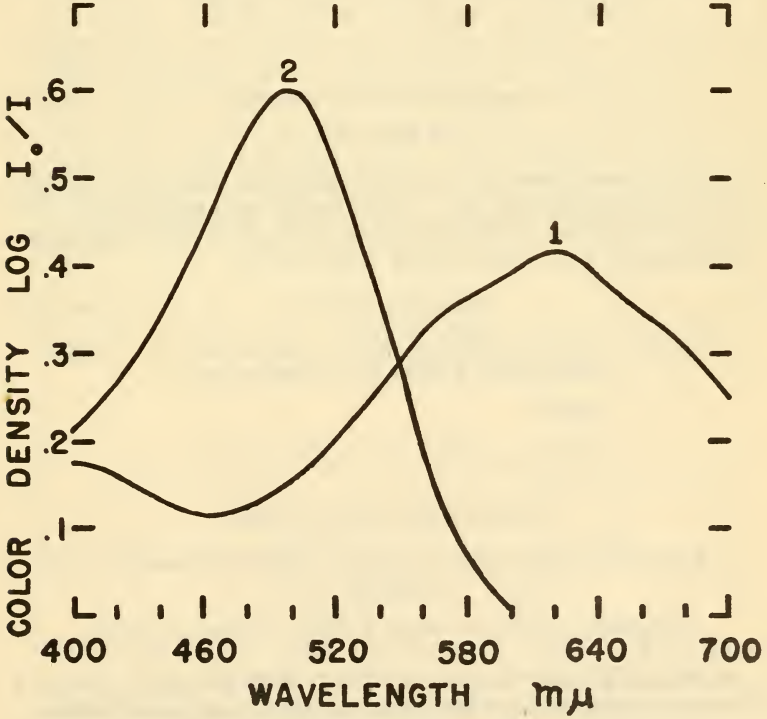


FIG. 8. Spectral curves of two azo dyes:

1. Janus green. 2. Congo red.

sections of embryos, and has been employed in the vital staining of fungi and protozoa; also the supravital staining of blood (Sabin, 1929).

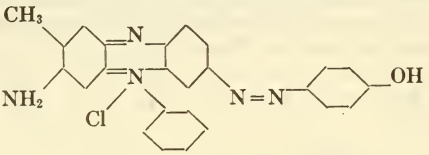
Soep (1927) proposes it in place of methylene blue for studying reductase production in milk.

A spectrophotometric curve of this dye is given in graph 1, Fig. 8, p. 79. Like other green dyes, it has two absorption maxima, one in the violet and the other near the orange range.

For use in **Supravital staining of blood,** see Staining Procedures, p. ID_3-9.
For use in **Lillie modification of Ziehl-Neelsen technic,** see Id., p. $IIIB_3$-11.

c52 JANUS BLACK C. I. NO. 134

Synonym: *Diazine black.*



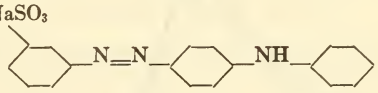
$C_{25}H_{20}N_5OCl$; *Mol. Wt.* 441.907

(*A basic dye*)

It will be seen from the above formula that this dye, like Janus green B, is an azo-safranin. It has rarely been called for in biology; but according to Maheshwari and Wulff (1937), Brestlavetz has employed it in vital staining pollen tubes.

c58 METANIL YELLOW C. I. NO. 138

Synonyms: *Orange MNO or MN. Acid yellow R. Soluble yellow OL. Yellow M. Tropaeolin G.*



$C_{18}H_{14}N_3O_3SNa$; *Mol. Wt.* 375.373

(*An acid dye; absorpiton maximum 536 in hydrochloric acid solution*)

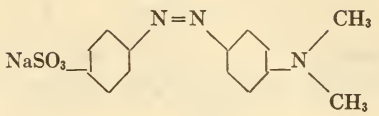
Solubility at 26°C: in water 5.36%; in alcohol 1.45%

In Masson's (1929) technic (see Foot, 1933) this dye is used as a connective tissue stain following hematoxylin and acid fuchsin.

For a technic adapted from Masson (1923), calling for **Mucicarmine with hematoxylin and metanil yellow,** see Staining Procedures, p. IA_4-7.

c60 METHYL ORANGE C. I. NO. 142

Synonyms: *Orange III. Helianthin. Gold orange MP. Tropaeolin D.*



$C_{14}H_{14}N_3O_3SNa$; *Mol. Wt.* 327.333

(*A weakly acid dye; absorption maximum 506–507 in acid solution*)

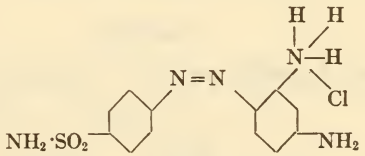
Solubility at 26°C: in water 0.52%; in alcohol 0.08%

This dye has little use as a stain, but is widely employed as an indicator, as it is red in acid, and orange in alkaline solutions. Its chief value as an indicator is that it is sensitive to mineral acids without being affected by carbonates or most organic acids. It has been used as an indicator for cell sap by Pfeffer (see Krause, p. 2305), and occasionally as a counterstain in histology. Under the name of gold orange, it has been employed by Newcomer (1938) as a counterstain to crystal violet in staining pollen tubes.

A spectral curve of this dye, compared with orange II and orange G, is given in Fig. 7, p. 74.

c64 PRONTOSIL

Synonym: *Prontosil red.*

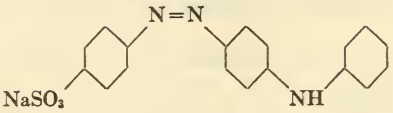


$C_{12}H_{14}N_5O_2SCl$; *Mol. Wt.* 327.789

This is a red substance employed principally as a therapeutic agent. It is essentially a basic dye in its chemical structure, although not used as a dye. It is referred to here only because it has been called for by Carter (1930) as a vital stain for insects and plants.

c68 ORANGE IV C. I. NO. 143

Synonyms: *Orange N. Acid yellow D. Tropaeolin OO.*



$C_{18}H_{14}N_3O_3SNa$; *Mol. Wt.* 375.373

(*An acid dye; absorption maximum about 527 in acid solution*)

Solubility at 26°C: in water 0.16%; in alcohol 0.2%

The only biological use of this dye seems to be occasionally as an indicator.

c71 BRILLIANT YELLOW S C. I. NO. 144

Synonyms: *Curcumine. Yellow WR.*

$C_{18}H_{13}N_3O_6S_2Na_2$; *Mol. Wt.* 477.422

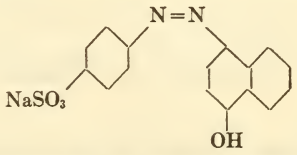
(*An acid dye*)

This dye is closely related to metanil yellow and orange IV, being manufactured by the sulfonation of the latter and containing one more sulfonic acid group. The exact position of the latter in the molecule is uncertain.

Margolena (1933b) has employed this dye in the Doglio (1932) technic, in which it is employed as a counterstain to carbol fuchsin in staining acid-fast bacteria. In Doglio's original paper the exact nature of the dye used is not specified, however, and at least two other dyes (C. I. Nos. 146 and 364) have been sold as brilliant yellow.

c75 ORANGE I C. I. NO. 150

Synonyms: *Naphthol orange. Tropaeolin G or 000 No. 1.*



$C_{16}H_{11}N_2O_4SNa$; *Mol. Wt.* 350.321

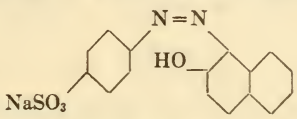
(*An acid dye; absorption maximum about 476*)

Solubility at 26°C: in water 5.17%; in alcohol 0.64%

This is another dye which is turned red by excess of alkali and has therefore some use as an indicator. McLean and Ireland (1940) have employed it with malachite green for staining sections of roots or stems of plants.

c76 ORANGE II C. I. NO. 151

Synonyms: *Gold orange. Orange A, P, or R. Acid orange II, Y or A. Orange extra. Mandarin G. Tropaeolin 000 No. 2.*



$C_{16}H_{11}N_2O_4SNa$; *Mol. Wt.* 350.321

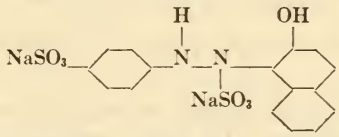
(*An acid dye; absorption maximum about 483–487*)

Solubility at 26°C: in water 11.37%; in alcohol 0.15%

This dye, which differs from orange I only in the position of the hydroxyl group on the naphthalene radical, is similar to it in color and properties, but does not change color with changing reaction of its solution. This dye is rarely employed in microscopic work, and yet may be recommended as a valuable substitute for orange G when a stronger yellow is desired for contrast purposes. Thus French (1926b) uses it in combination with eosin and azure C in a general tissue stain. It has been used by Bergonzini (1891) in place of orange G in the Ehrlich-Biondi stain; and by Ebbinghaus (1902) for staining keratin in sections of skin. Kalter (1943) includes it in a quadruple staining method, the other dyes in combination with it being fast green FCF, safranin O and crystal violet.

A spectral curve of this dye is given in graph 3, Fig. 7, p. 74.

c78 NARCEIN C. I. NO. 152



$C_{16}H_{12}N_2O_7S_2Na_2$; *Mol. Wt.* 454.386

(*An acid dye*)

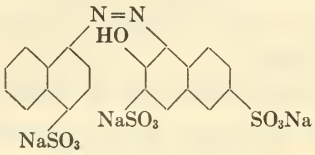
Solubility at 26°C: in water 10.02%; in alcohol 0.06%

This dye is a derivative of orange II, prepared from the latter by treatment with sodium bisulfite. It is rarely used either as a

textile dye or in microscopic technic. It has been called for by Ehrlich, however, in combination with pyronin and methyl green or methylene blue to form a neutral dye. It is no longer on the market, however.

c81 AMARANTH C. I. NO. 184

Synonyms: *Naphthol red S, C or O. Fast red. Bordeaux. Bordeaux SF. Victoria rubin O. Azo rubin. Wool red.*



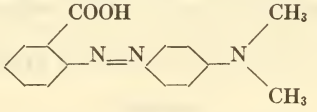
$C_{20}H_{11}N_2O_{10}S_3Na_3$; *Mol. Wt.* 604.475

(*An acid dye; absorption maximum about 525*)

Solubility at 26°C: in water 7.20%; in alcohol 0.01%

Amaranth is not a commonly used stain, but is of considerable importance as a food color. It has been used by Griesbach (1886) for staining axis cylinders, by Chambers (1935) in staining cells in tissue culture; also by Smith (1939) in color photomicrography.

c85 METHYL RED C. I. NO. 211



$C_{15}H_{15}N_3O_2$; *Mol. Wt.* 269.294

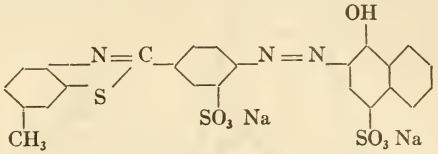
(*A weakly acid dye; absorption maximum 530*)

This dye has rarely been used for staining, but has long been employed as an indicator. Its useful range is from pH 4.4 to pH 6.0 in which it changes from red in acid solutions to yellow in basic. Although still of value for this purpose it is coming to be replaced by certain of the sulfonphthalein indicators such as brom cresol green and chlor cresol green (see page 203), which are more stable chemically and permit greater accuracy in reading. The chief drawback to methyl red as an indicator is that it is easily reduced with loss of color, and readings must be made very prompt-

ly after adding it to the solution to avoid error due to this cause. Carter (1933) has recently employed this dye in the vital staining of protozoa.

D1 THIAZINE RED R C. I. NO. 225

Synonym: *Chlorazol pink Y. Rosophenine 10B.*



$C_{24}H_{15}N_3O_7S_3Na_2$; *Mol. Wt.* 599.558

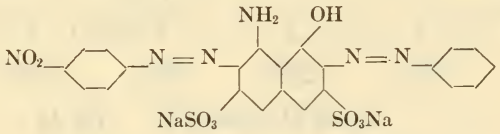
(*An acid dye; absorption maximum 505*)

This dye has occasionally been used as a counterstain in animal histology. It was introduced by M. Heidenhain (1903). It is thought by some users to prove a satisfactory counterstain against hematoxylin for skeletal and cardiac muscle.

b. Dis-Azo and Poly-Azo Dyes

D2 NAPHTHOL BLUE BLACK C. I. NO. 246

Synonyms: *Pontacyl blue-black SX. Buffalo black NBR.*



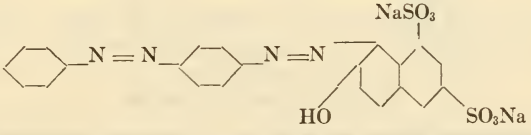
$C_{22}H_{14}N_6O_9S_2Na_2$; *Mol. Wt.* 616.505

(*An acid dye*)

This dye has rarely been mentioned for biological use; but it has been referred to by Lillie (1945c) as a stain for collagen. See Staining Procedures p. IB$_3$10.

D3 BRILLIANT CROCEINE C. I. NO. 252

Synonyms: *Croceine scarlet 3B, MOO.*



$C_{22}H_{13}N_4O_7S_2Na_2$; *Mol. Wt.* 555.482

(*An acid dye*)

Brilliant croceine has been mentioned by Lillie (1945c) as a substitute for "xylidine ponceau" in the Masson triple stain.

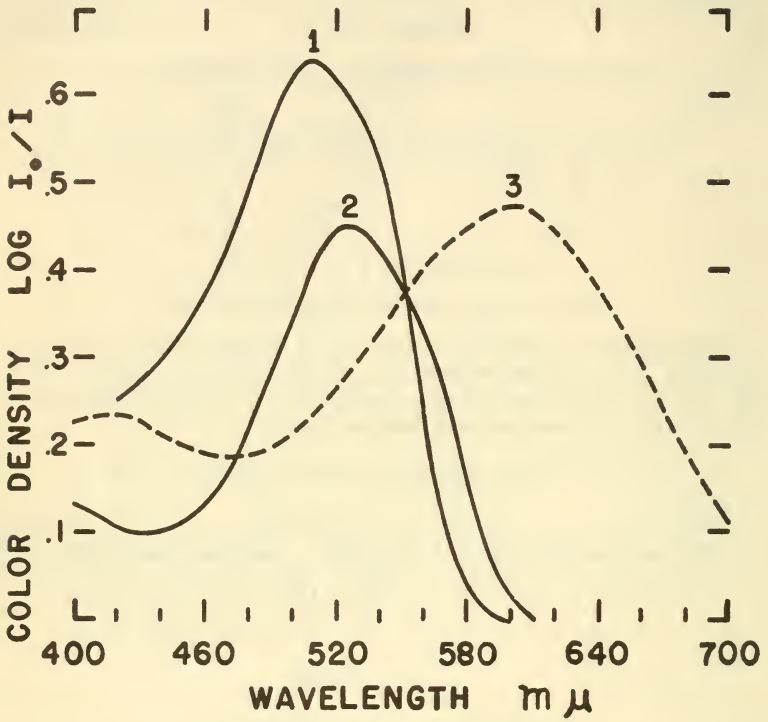
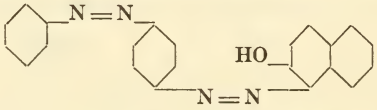


FIG. 9. Spectral curves of three azo dyes:
1. Sudan III. 2. Sudan IV. 3. Sudan black B.

D4 SUDAN III C. I. NO. 248

Synonyms: *Sudan G. Tony red. Scarlet B, fat soluble. Fat ponceau G. Oil red AS, O, B or 3B. Cerasin red.*



$C_{22}H_{16}N_4O$; *Mol. Wt.* 352.380

(*A weakly acid dye; absorption maxima in alcohol 508–510*)
Solubility at 26°C: in water nil; in alcohol 0.15%

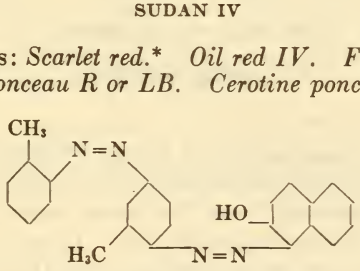
The chief value of this dye to the histologist is because of the position of the hydroxyl group, which as explained above (pp. 69, 76) makes it oil soluble and hence a fat stain. It was introduced as a fat stain by Daddi in 1896. Herxheimer (1901) investigated this and several other fat stains, proposing formulae which are still used as the basis of modern procedures. It is also employed by botanists together with light green in the technic of Bugnon, for differentiating suberized and cutinized tissue in plants.

For some time Sudan III was the only important fat stain known. More is now known in regard to fat soluble stains, thanks to the research of Michaelis (1901). It was he who showed the relation of this property of certain dyes to their lack of basic or acid character. He showed that new dyes with this property and of greater staining power might be built up synthetically by taking advantage of the fact that the azo group will attach itself in the ortho position if the para position is already occupied. In this way azo-ortho-phenols and beta-naphthols can be prepared, and they prove to be fat soluble. Michaelis suggested the following dye, which has now to a considerable extent replaced Sudan III.

Spectrophotometric graphs of Sudan III and Sudan IV are given in Fig. 9, page 86.

D6 SUDAN IV C. I. NO. 258

Synonyms: *Scarlet red.** *Oil red IV. Fat ponceau. Fat Ponceau R or LB. Cerotine ponceau 3B.*



$C_{24}H_{20}N_4O$; *Mol. Wt.* 380.432

(*A weakly acid dye; absorption maxima in alcohol 522–529*)

Solubility at 26°C: in water nil; in alcohol 0.09%

This di-azo naphthalene compound is similar to Sudan III except that it is a dimethyl derivative. This fact makes it a deeper, more intense stain; but having the hydroxyl group in the ortho

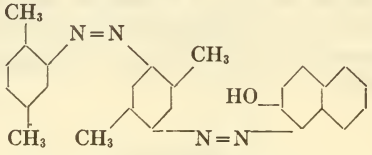
*Various erroneous variations of this term, some wholly or partly in German (e.g. Scarlet R, Scharlach R, Scharlach red) occur in the literature and on labels. They may be corruptions of the brand name, Biebrich scarlet R, Med.

position, it has similar physical properties and is fat soluble. Until recently it has been regarded as one of the best fat stains known; see, however, oil red O and oil red 4B, below, oil blue NA (p. 228) and Sudan black B, (p. 98).

For the technic of the **Herxheimer stain for fat** calling for the use of Sudan IV, see Staining Procedures, p. ID_3-18. For **Lillie's supersaturated isopropanol technic**, see p. ID_3-20.

D8 OIL RED O

As explained elsewhere, this dye has often been confused with Sudan II. It is, however, quite different in composition, more closely related to Sudan IV than to Sudan II. It consists of the following together with its isomers having the methyl groups in other positions:



$C_{26}H_{24}N_4O$; *Mol. Wt.* 408.484

(*A weakly acid dye*)

Oil red O was recommended by French (1926c) as a substitute for Sudan III, to be preferred because of its greater depth of color. Lillie (1944c) recommends it for this same reason, regarding it as one of the best fat stains known to him. It may be employed in the Herxheimer formula (an approximately 0.1% solution in equal parts of acetone and 70% alcohol), in the pyridin formula of Proescher (1927), or in Lillie's supersaturated isopropanol technic.

For **Proescher's oil-red-O-pyridin** technic, see Staining Procedures, p. ID_3-19.

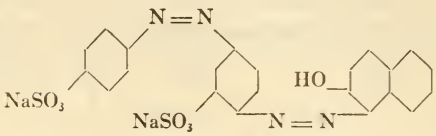
For **Lillie's supersaturated isopropanol technic**, see Staining Procedures, p. ID_3-20.

D10 OIL RED 4B

This is a dye of uncertain composition which is rarely referred to in the biological literature, but mentioned by Lillie (1944c) as one of the best fat stains to employ in the supersaturated isopropanol technic.

D15 BIEBRICH SCARLET, WATER SOLUBLE C. I. NO. 280

Synonyms: *Croceine scarlet. Scarlet B, or EC. Ponceau B. Double scarlet BSF.*



$C_{22}H_{14}N_4O_7S_2Na_2$; *Mol. Wt.* 556.478

(*An acid dye; absorption maximum about 503.5*)

Solubility at 26°C: in alcohol 0.05%

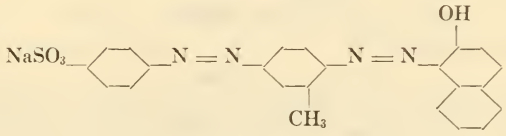
The chief biological application of this dye is for medicinal purposes, but it is occasionally used as a plasma stain, notably for tissues after staining with polychrome methylene blue or Unna's hematein. It has also been made use of by Paladino (1895) mixed with alum hematoxylin for double staining effect on histological material. In a neutral stain combination with ethyl violet, it has been employed by Bowie (1924) for staining the islets of Langerhans. More recently it has been recommended by Lillie (1940) in the Masson trichrome technic, by Schoor (1941) in combination with orange G and fast green FCF for staining vaginal smears; while McFarlane (1944) uses it in the modification of the Mallory stain which he terms "Picro-Mallory".

For use of **Biebrich scarlet with picro-aniline-blue,** or with **methyl blue,** see Staining Procedures, p. IB$_3$-12.

For technic of **Masson trichrome stain, Lillie modification,** see Id. p. IB$_3$-18.

D16 PONCEAU S. C. I. NO. 282

Synonym: *Fast ponceau 2B.*



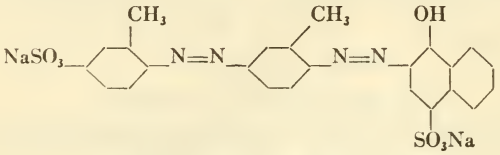
$C_{23}H_{17}N_4O_4SNa$; *Mol. Wt.* 468.282

(*An acid dye*)

Reference to this dye in biological literature shows a rather unusual history. It was mentioned quite early by Curtis (1905) who found it an improvement over acid fuchsin in the VanGieson stain.

Little attention was paid to the suggestion, however, until quite recently when the paper was discovered almost simultaneously by Ruth (1946) and Leach (1946) both of whom found the Curtis modification a useful one as it is less subject to fading.

D18 ORSEILLIN BB C. I. NO. 284



$C_{24}H_{18}N_4O_7S_2Na_2$; *Mol. Wt.* 584.530

(*An acid dye; absorption maximum 523*)

Cohen and Doak (1935) employed this dye in contrast to crystal violet for staining a mycorrhizal fungus on the roots of plants. Subsequently other authors including Alcorn and Yeager (1937), Keener (1951), have used it for staining other fungal parasites. Maneval (1941) lists it among several useful bacterial stains.

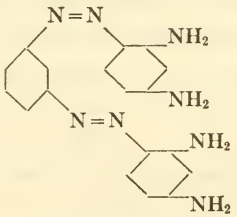
D21 BISMARCK BROWN Y C. I. NO. 331

Synonyms: *Vesuvin. Phenylene brown. Manchester brown. Excelsior brown. Leather brown. Basic brown G, GX, or GXP.*

Slightly different shade: *Bismarck brown G.*

(*A basic dye; absorption maximum 463. Solubility at 26°C: in water 1.36%; in alcohol 1.08%*)

The various shades of Bismarck brown are mixtures of different compounds, the most important of which are salts of the following:



The dihydrochloride is $C_{18}H_{20}N_8Cl_2$; *Mol. Wt.* 419.318

This dye was formerly employed quite extensively as a contrast stain, but has now been replaced to some extent by others. It is

still used, however, as a mucin stain, and is good for vital staining and for staining in bulk. It is employed in staining cellulose walls of plants in contrast to hematoxylin; and occasionally for staining bacteria in contrast to gentian violet in the Gram technic, to methyl or crystal violet in the Ljubinsky stain for diphtheria organisms, or to carbol-fuchsin for staining acid-fast bacteria. Blaydes (1939) points out that it stains more intensely if used in phenolic solution.

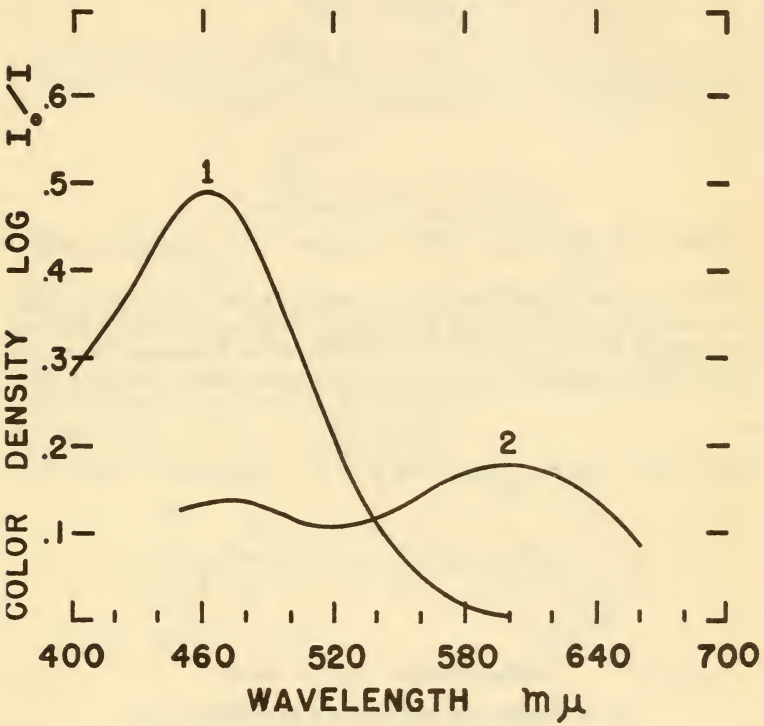


FIG. 10. Spectral curves of two azo dyes:
1. Bismarck brown Y. 2. Chlorazol black E.

A caution to observe in connection with Bismarck brown is that solutions should not be boiled before using, because the composition of the dye is changed by heat.

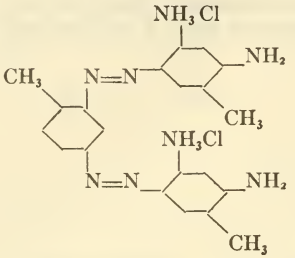
A spectrophotometric curve of this dye is given in graph 1, Fig. 10.

For technic as **mucin and cartilage stain in contrast to methyl green,** see Staining Procedures, p. IA_4-5.

For technic of **Ljubinsky stain for diphtheria,** see Id, $IIIA_2$-12.

D22 BISMARCK BROWN R C. I. NO. 332

Synonyms: *Bismarck brown GOOO 53A or 53B. Brown R, AT, C or N. Manchester brown EE. Vesuvin NR, B, R. Basic brown BR or BXN.*



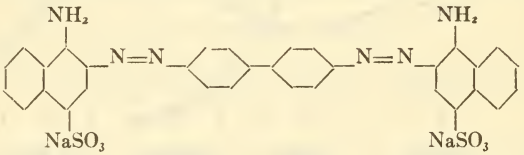
$C_{21}H_{26}N_8Cl_2$; *Mol. Wt.* 461.396

(*A basic dye. Solubility at 26°C: in water 1.10%, in alcohol 0.98%*)

This dye has not ordinarily been employed as a biological stain. One sample was submitted by a stain company by mistake, and it appeared to be a satisfactory substitute for Bismarck brown Y. Lillie (1945) specifies it as a good counterstain to oil blue NA.

D26 CONGO RED C. I. NO. 370

Synonyms: *Congo. Cotton red B or C. Direct red, C, R or Y.*



$C_{32}H_{22}N_6O_6S_2Na_2$; *Mol. Wt.* 696.658

(*An acid dye; absorption maximum about 497*)

Solubility at 26°C: in alcohol 0.19%

This dye is best known to the biologist as an indicator. The dye acid is blue, but its sodium salt is red. The red color of the salt is readily changed by weak acids into blue. Besides serving as an indicator, Congo red has certain histological uses, as for axis cylinders (Griesbach, 1886), for embryo sections (Schaffer, 1888), for staining elastic tissue (Matsuura, 1925), plant mucin, as a stain for Uredineae (Blackman, 1905), and as a general background stain in contrast to hematoxylin and other nuclear dyes. It has been used by Klebs (1886) as a reagent for cellulose. It proves

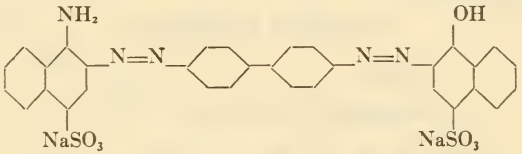
useful as a negative stain for bacteria (Benians, 1916, Cumley, 1935, Maneval, 1934) and for staining protozoa (Merton, 1932) and yeasts (Gutstein, 1932).

A spectrophotometric curve of this dye is given in graph 2, Fig. 8, page 79.

For use of **Congo red with Mayer's hemalum**, see Staining Procedures, IA_4-13.
For use in **Bennhold's stain for amyloid**, see p. IA_4-10.

D31 ERIE GARNET B C. I. NO. 375

Synonyms: *Congo corinth G or GW. Corinth brown G. Cotton corinth G. Amanil garnet H. Direct garnet R. Buffalo garnet R. Direct violet C. Diamine Bordeaux CGN.*

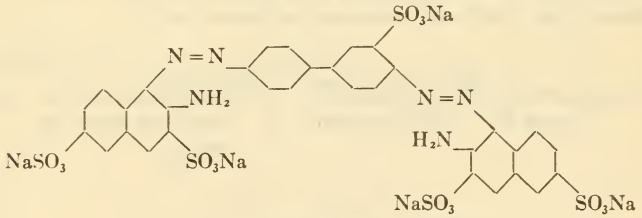


$C_{32}H_{21}N_5O_7S_2Na_2$; *Mol. Wt.* 697.642

(*An acid dye*)

This dye has been employed by Geschickter (1930b), mixed with azure A, for staining frozen fresh tissue.

D35 TRYPAN RED C. I. NO. 438



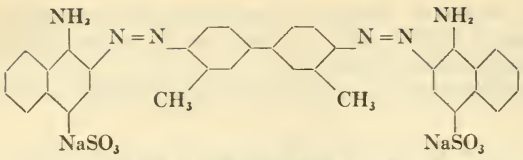
$C_{32}H_{19}N_6O_{15}S_5Na_5$; *Mol. Wt.* 1002.805

(*An acid dye*)

The chief use of this dye is as a vital stain. Varco and Vischer (1941), for instance, thus use it in studies of gastric secretion. As pointed out by Whitby (1942) this dye has an interesting history because it had very early application as a chemotherapeutic agent, and led ultimately to the discovery of the arsenicals.

D38 BENZOPURPURIN 4B C. I. NO. 448

Synonyms: *Cotton red 4B. Dianil red 4B. Diamin red 4B. Sultan 4B. Direct red 4B.*



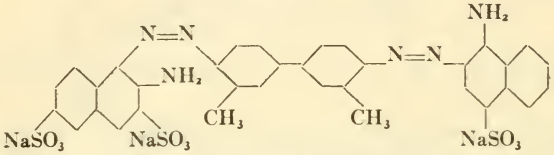
$C_{34}H_{26}N_6O_6S_2Na_2$; *Mol. Wt.* 724.710

(*An acid dye; absorption maximum about 497*)

Solubility at 26°C: in alcohol 0.13%

This dye has been employed by Zschokke (1888) as a plasma stain especially in contrast to hematoxylin, and by Ono (1934) for staining spirochaetes.

D41 BRILLIANT PURPURIN R C. I. NO. 454



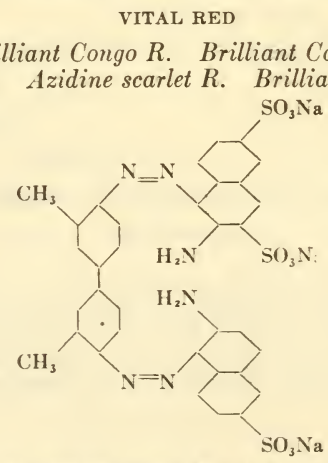
$C_{34}H_{25}N_6O_9S_3Na_3$; *Mol. Wt.* 826.759

(*An acid dye*)

Gutstein (1932) has employed a dye which he designates "brilliant purpur R" as a vital stain for yeasts. It probably is the above. Lillie (1948) includes this dye, with azo fuchsin G and naphthol blue black, in a connective tissue stain.

D45 VITAL RED C. I. NO. 456

Synonyms: *Brilliant Congo R. Brilliant Congo red R. Acid Congo R. Azidine scarlet R. Brilliant vital red.*



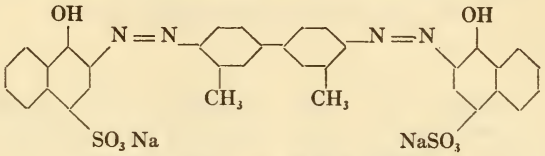
$C_{34}H_{25}N_6O_9S_3Na_3$; *Mol. Wt.* 826.759

(*An acid dye; absorption maximum about 498*)

This dye is a very important vital stain, much used by Evans. (See Dawson, Evans and Whipple, 1920.) Commercially it is better known under the name of brilliant Congo R. Difficulty was at first found in securing a satisfactory product; but it was overcome thanks to the coöperation of the Dept. of Agriculture, and a good vital red is now obtainable from stain dealers.

D50 AZO BLUE C. I. NO. 463

Synonym: *Benzoin blue R. Direct violet B.*



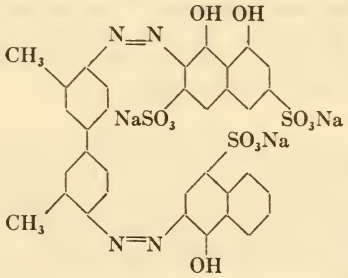
$C_{34}H_{24}N_4O_8S_2Na_2$; *Mol. Wt.* 726.678

(*An acid dye; absorption maximum 560.5*)

Monné (1935) has listed this dye among a series with which vital staining of protozoa has been secured. Butt, Bonynge and Joyce (1936) find that it can replace India ink in demonstrating capsules of bacteria. Broda (1939) uses it in the histochemical demonstration of magnesium.

D52 DIANIL BLUE 2R C. I. NO. 465

Synonyms: *Direct steel blue BB. Benzo new blue 2B. Naphthamine brilliant blue 2R.*



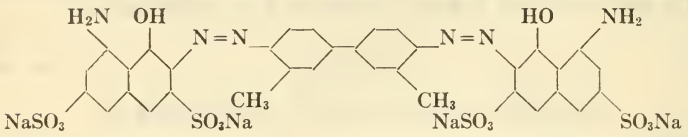
$C_{34}H_{23}N_4O_{12}S_3Na_3$; *Mol. Wt.* 844.727

(*An acid dye; absorption maximum about 568*)

This has been used as a vital stain by Corner and Hurni (1918); also by Sutter (1919), and Duran-Reynals (1937).

D55 TRYPAN BLUE C. I. NO. 477

Synonyms: *Chlorazol blue 3B. Benzo blue 3B. Dianil blue H3G. Congo blue 3B. Naphthamine blue 3BX. Benzamine blue 3B. Azidine blue 3B. Niagara blue 3B.*

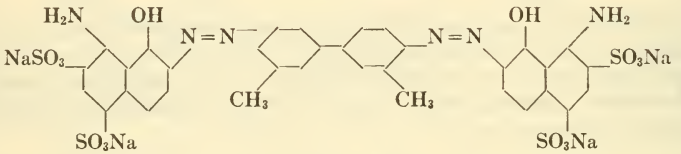


$C_{34}H_{24}N_6O_{14}S_4Na_4$; *Mol. Wt.* 960.808

(*An acid dye*)

This is a valuable vital stain. By injection into the circulatory system, it is employed for staining the uriniferous tubules; also for numerous other purposes in the vital staining of various kinds of tissue, from mammals, lower vertebrates and insects.

D56 EVANS BLUE

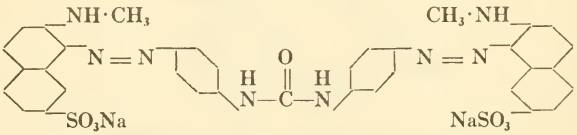


$C_{34}H_{24}N_6O_{14}S_4Na_4$; *Mol. Wt.* 960.808

(*An acid dye*)

The similarity between Trypan blue and Evans blue is evident by a glance at the two formulae. The latter, like the former, is employed as a vital stain. It has been thus employed in several rather varied applications: see Brunschwig, Schmitz and Jennings (1940), and Zahl and Waters (1941).

D60 VITAL NEW RED



$C_{35}H_{28}N_8O_7S_2Na_2$; *Mol. Wt.* 782.752

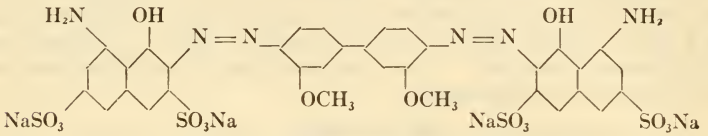
(*An acid dye*)

No reference can be found to the manufacture of this dye except in England. It is not listed in dye indexes, the dye most nearly

like it thus recognized being C. I. No. 353 (chlorazol fast pink 4BL) which differs from it primarily in that it is not methylated, has two hydroxyl groups attached to the naphthalene rings, and two sulfonic groups attached to the benzene rings. It has been called for in certain vital staining procedures, having been introduced for that purpose by Evans and Scott in 1921.

D65 NIAGARA BLUE 4B C. I. NO. 520

Synonyms: *Pontamine sky blue 5BX. Direct sky blue. Benzo sky blue.*



$C_{34}H_{24}N_6O_{16}S_4Na_4$; *Mol. Wt.* 992.808

(*An acid dye; absorption maxima about 634.2 and 589.5*)

Occasional references have been made to this dye in connection with histology for some time, although it has never come into general use. Recently Varrelman (1938) has employed it for demonstrating the vascular system of plants, taking advantage of its rise through stems which are stood in a solution of the dye. In the vital staining of skin or of tumors a pontamine sky blue has been recommended, which may be this dye or may be C. I. No. 518 which has been sold under the name of pontamine sky blue 6BX (Syn. *Niagara sky blue* 6B or *direct sky blue*).

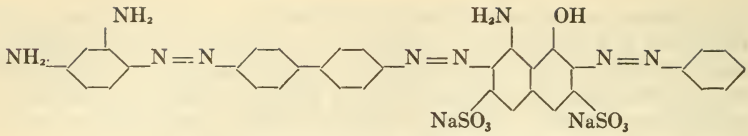
D68 MARSHALL RED

D70 HICKSON PURPLE

These are two new dyes of unknown composition, but stated to belong to the disazo group. They are discussed by Cannon (1941) as having been manufactured in the course of his study of chlorazol black E and of dyes of other colors found to be associated with that dye. He claims that they are valuable nuclear stains in general histology.

D75 CHLORAZOL BLACK E C. I. NO. 581

Synonyms: *Pontamine black E, EX,* or *EXX. Erie black GXOO, B, BF. Direct black MS, RL, E, GX, EE, 2V, F or A. Direct deep black EW extra, E, EA, EAD extra. Renol black G.*



$C_{34}H_{25}N_9O_7S_2Na_2$; *Mol. Wt.* 781.726

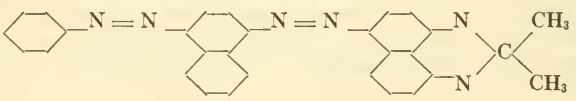
(*An acid dye; absorption maximum 598–602 in alcohol.*)

This dye has only recently been introduced into microscopic technic, Cannon (1937) having called attention to its use in general histology and cytology. It proves to be a valuable stain because it can be employed without mordanting or differentiation and gives a very sharp clear-cut picture both of nuclear and cytoplasmic structures. The black and gray tones it produces are well suited for photography.

For use as **general stain in animal histology**, see Staining Procedures, p. $1A_4$-9.
For use as **general stain in plant histology**, see Id. p. IIA-6.
For use as **plant cytological stain with aceto-carmine**, see Id. p. IIB-4.

A spectral curve of this dye is given in graph 2, Fig. 10, p. 91. It will be noticed that the black nature of the solution is indicated by partial absorption through the whole visual range, with only minor peaks at any wavelength.

D80 SUDAN BLACK B



$C_{29}H_{22}N_6$; *Mol. Wt.* 554.414

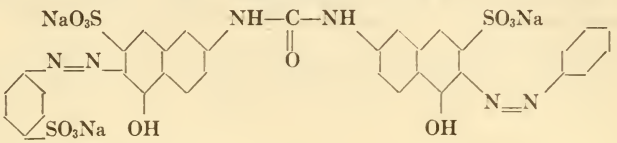
(*Absorption maximum 596–605.*)

This compound is one of the most recently developed fat stains. For a fat stain it has a rather unusual chemical structure, as it does not show the typical -OH group in the ortho (or beta) position. It was first prepared in Germany some time in the early '30's, and became available in France and England. Lison and Dagnelie (1935) proposed it as a myelin stain; but it was in England that its value as a stain for fats was first developed (Gerard, 1935). During the second World War it achieved general recognition for the purpose, both in Great Britain and in the United States. Hartman (1940) and later Burdon *et al.* (1942) found it valuable as a stain for bacterial fat. It has also been proposed for staining leucocyte granules and the Golgi apparatus.

Unfortunately just at the time when its value was most appreciated it went off the market entirely because of the war. It was not till after the close of the war that American manufacturers learned its formula and discovered how to make it.

A spectral curve of this dye is given in graph 3, Fig. 9, p. 86. Characteristic of a black dye is the fact that there is considerable light absorption even at the curve's minimum.

D82 ORANGE S



(*An acid dye*)

Occasional reference has been made to an orange S in the biological literature, which we are informed by the dye industry is probably of the above composition. It was proposed by DeGalantha (1936) in a mixture with alum hematoxylin for staining connective tissue and following Harris' hematoxylin for demonstrating amyloid.

D85 VICTORIA GREEN G

A new trisazo dye of unknown chemical composition studied by Cannon (1941) in connection with other new azo dyes. He states it to have valuable properties as a nuclear stain in general histology.

Other azo dyes sometimes mentioned in connection with histology are:

Janus red; C. I. No. 266.

Tropaeolin O; C. I. No. 148. Syn: *Chrysoin. Gold yellow. Acme yellow.*

Tropaeolin Y; C. I. No. 148 (see note).

Roccellin; C. I. No. 176. Syn: *Fast red A, AV, AL, BX, S* or *O. Cerasin. Rubidin. Cardinal red.*

Crystal ponceau 6R; C. I. No. 89; Syn: *Ponceau 6R.*

Carmine naphtha; C. I. No. 23. Syn: *Sudan G. Oil yellow.*

Alizarin yellow GG; C. I. No. 36. Syn: *Anthracene yellow GG. Mordant yellow 2GT.*

Chlorazol paper brown B. A dye recently put out by the Imperial Chemical Industries of Manchester, England. Proposed by Verdcourt (1947) for staining plant tissues.

Chrysoidin R; C. I. No. 21. Syn: *Cotton orange. Cerotin orange.*

Alizarin yellow R; C. I. No. 40. Syn: *Alizarin orange. Mordant yellow PN. Orange R. Anthracene yellow RN.*

Diamond flavine G; C. I. No. 110.

Diamond black F; C. I. No. 299. Syn: *Salicin black D. Chrome black J or F.*

Indole blue B or R; C. I. No. 135. Syn: *Indoine blue BB, R* or *BR. Safranin blue R. Janus blue G* or *R. Indophene blue. Basilene fast blue B.*

Direct violet K, N or R; C. I. No. 394. Syn: *Chlorazol violet N. Pontamine violet N. Diamine violet N. Erie violet 3R.*

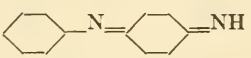
CHAPTER VI

THE QUINONE-IMINE DYES

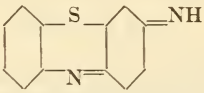
THE dyes of the quinone-imine group contain two chromophore groups, the indamin group —N=, and the quinoid benzene ring

They are derivatives of the theoretical compound paraquinone-di-imine, which, if it existed in its free state, would have the formula

In the typical indamin formula one of the imine hydrogen atoms is replaced by a phenyl group, thus:

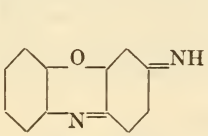


In the thiazins the introduction of a sulfur atom, attached to both the phenyl and the quinone groups, forms a third closed ring, as:

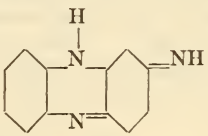


imino-thio-diphenylimine

In the oxazins an oxygen atom, and in the azins a nitrogen atom takes the place of the sulfur of the thiazins, thus:



oxazin



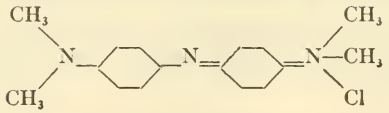
azin

1. THE INDAMINS

The indamin dyes are methylated amino derivatives of indamin. No dye in this group is a common biological stain. The

following are occasionally mentioned, however, in connection with histology:

H5 BINDSCHEDLER'S GREEN C. I. NO. 819

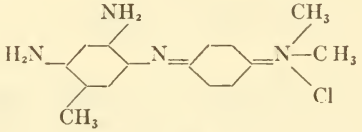


$C_{16}H_{20}N_3Cl$; *Mol. Wt.* 289.801

(*A basic dye; typically of the above formula, but sometimes sold as the zinc double salt*)

Bindschedler's green has occasional employment as an indicator of oxidation reduction. It is reduced to the colorless *pp'*-tetramethyldiamino-diphenylamine. Elliott and Baker (1935) have recently employed it for this purpose in studying the metabolism of tumor tissue.

H10 TOLUYLENE BLUE C. I. NO. 820



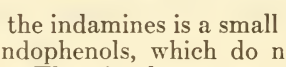
$C_{15}H_{19}N_4Cl$; *Mol. Wt.* 290.791

(*A basic dye*)

Like its homolog, Bindschedler's green, toluylene blue is readily reduced to a substituted diphenylamine; and accordingly it has similar indicator properties.

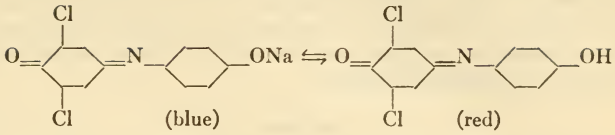
2. THE INDOPHENOLS

Closely related to the indamines is a small group of colored compounds known as indophenols, which do not readily form salts with mineral acids. The simplest compound of this group is indophenol (sometimes called benzenoneindophenol) and has the following structural formula.

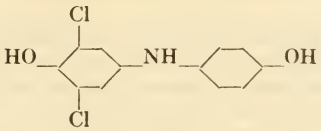


Various halogen substitution products of indophenol are known, and all have indicator value, showing an acid-base color change and a disappearance of color on reduction. The best known indicator of this series is:

15 2, 6-DICHLOROINDOPHENOL



which on reduction becomes the following colorless compound:

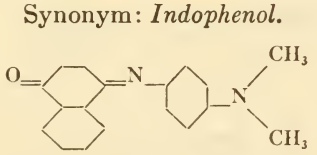


It will be seen that the type of color change shown by this indicator is very similar to that of litmus (see p. 217). Litmus is a non-synthetic product of unknown chemical composition; it is not believed to be related to the indophenols, in spite of its similar behavior as an acid-base and oxidation-reduction indicator.

There is only one other dye of this group that need be mentioned here.

120 INDOPHENOL BLUE C. I. NO .821

Synonym: *Indophenol.*



$C_{18}H_{16}N_2O$; *Mol. Wt.* 276.324

(*Absorption maximum in alcohol about 592*)

This compound is occasionally referred to in biological work. Herxheimer (1901), for example, employed a dye which he called indophenol (and which probably was indophenol blue), in saturated solution in 70% alcohol, as a fat stain. It is still sometimes used for the same purpose; see Black (1938), for instance.

3. THE THIAZINS

The thiazins constitute one of the most important groups of dyes from the standpoint of the biologist; while for textile dyeing the group contains but a small number of dyes of any importance. In these compounds, as mentioned above, the two benzene rings are further joined by a sulfur atom.

Considerable information is at hand concerning the spectro-

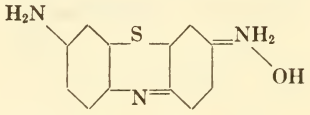
photometric characteristics of the thiazins (Knapp, Emery and Stotz, 1950). From this information the absorption curves given in Figs. 11 and 12 have been derived for seven of the dyes in the group. Reference to these curves will be given under the individual dyes as discussed below.

J5 THIONIN C. I. NO. 920

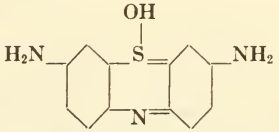
Synonym: *Lauth's violet.*

Thionin, having two amino groups, is a strongly basic dye. The exact structural formulae of this dye and its derivatives, as well as many others in which two benzene rings are similarly joined, are in some dispute. At least two types of formulae are possible for the thiazins and oxazins, as well as for the xanthene dyes (Chapter VIII). One type is known as the orthoquinoid, the other as the paraquinoid.

It is supposed (see p. 25) that when the quinoid ring is formed the two hydrogen atoms replaced by atoms or groups with double valency bonds may be either in the para or in the ortho position to each other. It will also be recalled from elementary chemistry that sulfur and oxygen may be either bivalent or tetravalent. These facts make it possible for a thiazin or an oxazin to have either one or the other of the different structures represented by the following two formulae for the theoretical thionin base:



paraquinoid formula



orthoquinoid formula

In the case of the paraquinoid formula the compound is an ammonium base of the type discussed on p. 27, which is capable of salt formation through its pentavalent nitrogen. In the case of the orthoquinoid formula the salt formation takes place through the tetravalent sulfur, the base being of the type known as a sulfonium base. There are arguments in favor of either formula, and from the standpoint of the biologist it does not matter which is preferred. Possibly both forms actually exist simultaneously. For

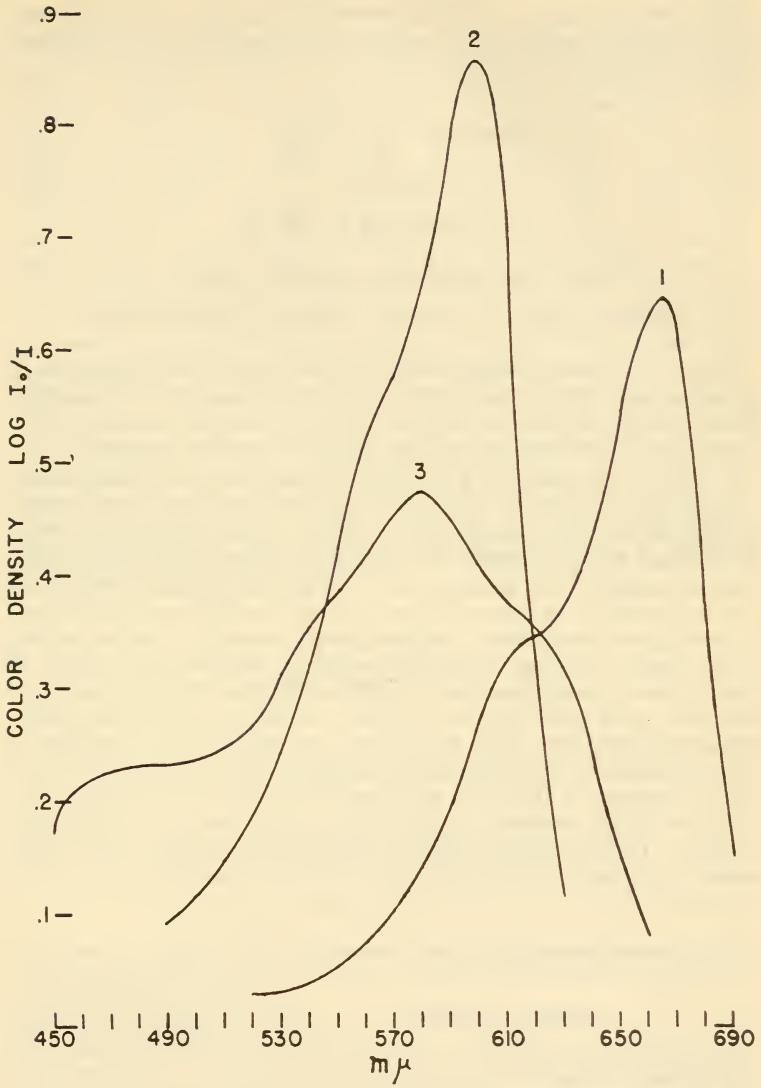
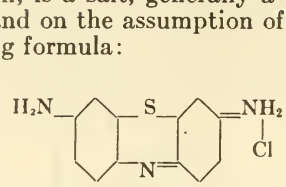


FIG. 11. Spectral curves of three thiazin dyes:

1. Methylene blue. 2. Thionin. 3. Methylene violet.

the sake of uniformity the paraquinoid form will be shown in the following pages wherever possible; but with the understanding that the orthoquinoid form is equally permissible.

The dye, thionin, is a salt, generally a chloride, of the above-mentioned base; and on the assumption of paraquinoid structure, it has the following formula:



$C_{12}H_{10}N_3SCl$; *Mol. Wt.* 263.741

(*A basic dye; absorption maximum 598–599.*)

Solubility at 26°C: in water 0.25%; in alcohol 0.25%

Thionin is violet in dilute solution, and has a very pronounced metachromatic effect, the colors in sections stained with it ranging from blue to reddish violet. A spectrophotometric graph of a typical sample of thionin is given in Fig. 11, p. 105 (curve 2).

It is no longer used as a textile dye, and is to be distinguished from thionin blue (C. I. No. 926) which is known to the trade and has sometimes been furnished in place of the desired dye when thionin has been ordered. Thionin is an especially valuable dye for histological work on account of its metachromatic properties, that is, its ability to impart different colors to different histological or cytological structures. It is a very valuable chromatin and mucin stain, proving especially useful in staining the tissue of insects; and is recommended by Ehrlich because it stains amyloid blue but mast cells and mucin red. It is a useful vital stain. Perhaps its greatest value at the present time is in the staining of frozen sections of fresh animal or human tissue, particularly in the study of tumors. It is also used by Frost (1916) for staining very young bacterial colonies in his "little plate" technic for counting bacteria. (Unfortunately Frost specified thionin blue in one of his papers, although the latter proves entirely unsatisfactory for the purpose.)

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED*

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Staining frozen sections	IA_4-18
Staining fixed tissue	IA_4-19
End point staining of Nissl granules	IC_3-14
Schmorl's method for staining bone sections	ID_3-12
Stoughton's technic (with orange G) for fungi in plant tissue	IIA-12

*Under this heading are given references to procedures described in detail in STAINING PROCEDURES, edited by Conn and Darrow (1943-52).

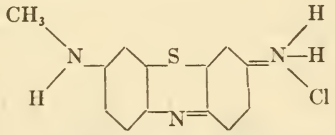
METHYLENE AZURE C. I. NO. 923

This was first recognized as one of the components of polychrome methylene blue by Bernthsen (1885). It is now known to be a mixture, not a simple dye. Definite knowledge of its chemistry has been obtained by Kehrmann (1906), Bernthsen (1906) and MacNeal (1906, 1925). It is regarded as primarily a mixture of azure A and azure B, described below, compounds which Bernthsen and MacNeal have shown simple methods for preparing in relatively pure condition by the oxidation of methylene blue. Another oxidation product of methylene blue, described later by Holmes and French (1926), has been named azure C. Now that these three dyes, azures A, B and C are recognized, it would be most satisfactory if the name methylene azure could be dropped, and users specify the actual dye employed.

Azure I (Giemsa) is a trade name applied to a secret preparation which appears to be a somewhat variable mixture in which azure B predominates. Azure II (Giemsa) is an intentional mixture of azure I with an equal quantity of methylene blue. Products of these same names are now sold by various companies and they are certainly not all identical with the original Giemsa preparations. They all probably agree, however, in containing azure A and azure B as their chief constituents.

j6 AZURE C

This dye is mono-methyl thionin:



$C_{13}H_{12}N_3SCl$; *Mol. Wt.* 277.767

(*A basic dye; absorption maximum 607–610*)

Curve 4 in Fig. 12, p. 108, is a spectrophotometric graph of a typical sample of azure C.

It is not a textile dye; and up to the present has been manufactured only on a laboratory scale. A small lot, thus prepared, has been put on sale by one of the American stain companies. This dye, like the other azures described below, has up to the present been prepared only by the oxidation of methylene blue. If they prove sufficiently valuable, a process of manufacture by direct synthesis will undoubtedly be developed. A product thus manufactured would probably be cheaper as well as purer than the present supply.

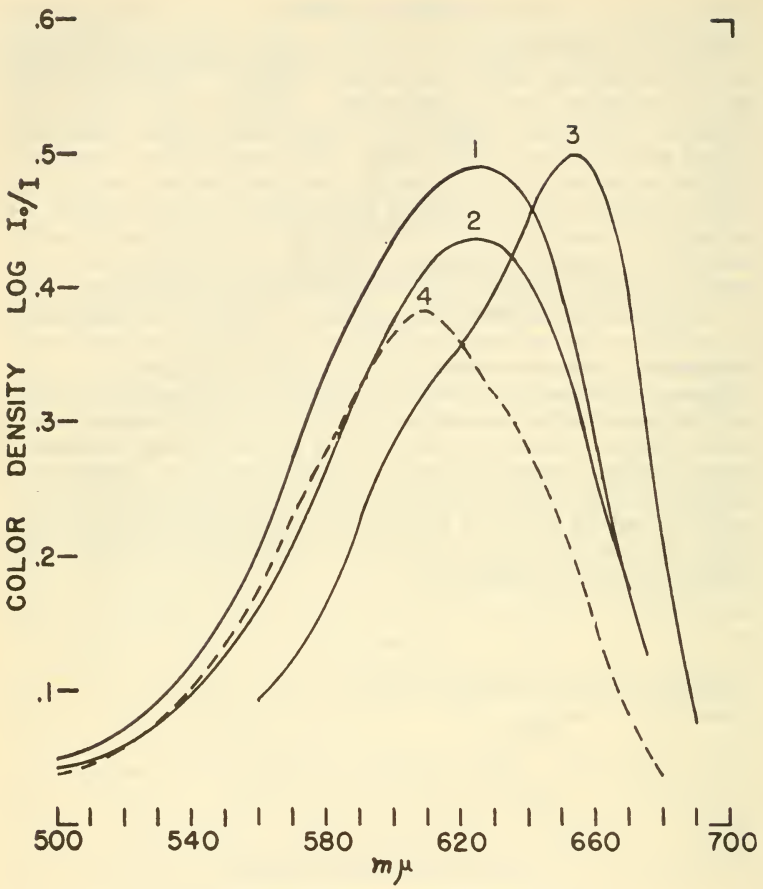


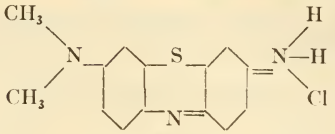
FIG. 12. Spectral curves of four thiazin dyes:

1. Toluidine blue.
2. Azure A.
3. Azure B.
4. Azure C.

Azure C is recommended by French (1926b) with orange II and eosin as a tissue stain. Haynes (1926a) finds it possible to obtain equally good results, however, with azure I (probably azure A) if the technic is slightly modified. Later (1927) she reports excellent staining with azure C; but it is still uncertain whether it gives results sufficiently different from thionin on the one hand and azure A on the other to be of decided histological value.

J7 AZURE A

This is now recognized as asymmetrical dimethyl thionin:

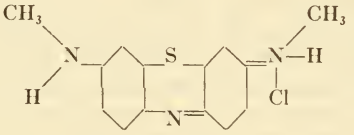


$C_{14}H_{14}N_3SCl$; *Mol. Wt.* 291.793

(*A basic dye; absorption maximum 625–632*)

Curve 2 in Fig. 12, p. 108, is a spectrophotometric graph of a typical sample of Azure A. (Its similarity to toluidine blue is well brought out in these curves.)

It is not a textile dye; but is probably present in much of the methylene blue on the market. It must be distinguished from the isomeric symmetrical compound:



which has never been given a special name. Ever since first recognized by Kehrmann (1906) the impression has been current that this latter dye has no staining value and is of a distinctly different category from the azures. It is difficult to tell how this impression has arisen, but possibly it may have been due to work with an impure dye. Haynes (1927) found both isomers to have almost identical staining properties.

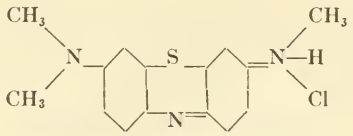
There is no mistaking the value of azure A; it was in fact regarded by MacNeal (1925) as the most important nuclear staining constituent of polychrome methylene blue. It was called for in the originally proposed formulae for the tetrachrome blood stain (see p. 244). It has been certified by the Commission for some time. Until recently it has been recommended for all purposes for which methylene azure or Azure I was specified; recent work, however, suggests that there are other constituents of the German Azure I (particularly azure B) which may have special value in blood stains.

Haynes (1926a) employed this dye as a nuclear stain preceding eosin; and (1926b) following phloxine in a procedure similar to the Mallory phloxine-methylene-blue technic. Douglas (1932) mixed it with methylene blue for a counterstain to acid-fast bacteria.

For use in **buffered azure-eosinate method** see Staining Procedures, p. IA_4-17; in Kinyoun's diphtheria stain, see p. $IIIA_2$-12.

J9 AZURE B

Azure B is the tri-methyl derivative of thionin:



$C_{15}H_{16}N_3SCl$; *Mol. Wt.* 305.819

(*A basic dye; absorption maximum 648–653*)

Like azure A, this compound was first recognized by Kehrmann (1906) as a constituent of polychrome methylene blue. It can easily be prepared in impure form by oxidation of methylene blue, and is now available if anyone desires to obtain it.

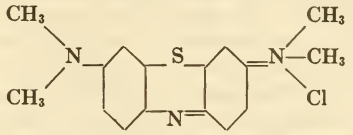
Curve 3 in Fig. 12, p. 108, shows its spectral characteristics. It is distinctly less red than any of the other azures.

MacNeal (1925) stated that its staining effects were much the same as could be obtained with a mixture of azure A and methylene blue, and that it therefore could be of little importance in polychrome methylene blue. His statement has generally been interpreted as meaning that azure B is not a satisfactory stain. Holmes and French (1926) also stated that this dye has little staining value; but their conclusions were based upon work with an impure sample, the performance of which was so poor as not apparently to justify further purification. Haynes' later work (1927) on sections of fixed tissue, with this same sample of azure B, agreed partially with this, although she found the dye, if used in buffered solution, rather better than fairly pure methylene blue in fresh solution. Jordan and Heather (1929) definitely specify this dye in a stain for Negri bodies. Moreover, recent tests by the Stain Commission of certain exceptionally good Giemsa stains indicate the possible presence of more azure B than in less satisfactory batches. Roe, Lillie, and Wilcox (1940), in fact, have concluded that azure B is one of the most important constituents of Giemsa stain, especially when used for staining malaria parasites in thick blood films. This work has been followed up by a series of other papers by Lillie and his associates, and they have distributed samples to other laboratories for testing; as a result it seems difficult to question their conclusions. Accordingly it is now recognized that Azure B is one of the most important constituents of "Azure I".

Synonym: *Swiss blue.*

Various grades denoted: *Methylene blue BX, B, BG, BB;* grade preferred for biological work: *Methylene blue chloride.*

Methylene blue is theoretically tetra-methyl thionin:



$C_{16}H_{18}N_3SCl$; *Mol. Wt.* 319.845

(*A basic dye; absorption maximum, if pure, about 664–666*)

Solubility at 26°C: in water 3.55%; in alcohol 1.48%

The dye is so readily oxidized that it is practically impossible to obtain in pure form, the presence of some of the azures or of methylene violet being almost universal. Methylene blue has long been recognized as an important nuclear stain. It is notable, however, that all the staining solutions recommended for use on sections of fixed tissue call for some ripening or for the addition of alkali. Such solutions are bound to contain one or more of the azures. There are indications, in fact, that pure methylene blue is a relatively poor histological stain.

A spectrophotometric graph of a reasonably pure sample of methylene blue is given in curve 1, Fig. 11, p. 105. It is to be noticed that this curve has a distinct "hump" at about 610 mμ; this is characteristic of methylene blue and may indicate the presence of either a tautomeric form or of some product of oxidation. It is interesting to observe that 610 is practically the absorption maximum of azure C.

The methylene blue of commerce is generally a double salt, the chloride of zinc and methylene blue. The zinc is toxic, however; so for some time the zinc-free methylene blue chloride has been prescribed for medicinal purposes, and is frequently designated methylene blue Med. U. S. P. The zinc double salt is less soluble, particularly in alcohol, so for most staining purposes is less desirable. The investigations of the Commission show that for all ordinary staining purposes the zinc-free compound is best; so that is the form at present recommended.

Methylene blue is perhaps the stain which the pathologist and bacteriologist would have the greatest difficulty in doing without, and it is of great value to the zoologist as well. It is employed for a greater variety of purposes than any other biological stain except

possibly hematoxylin; and for this reason was the first dye to be given a thorough investigation by the Commission. It is used: first, as a nuclear stain in histology, for which purpose its strongly basic character as well as the ease with which it can be applied without over-staining, make it quite valuable; second, as a bacterial stain, notably in milk work and in the diagnosis of diphtheria, where it is especially useful because it has an affinity for the bacterial protoplasm as great as that of the rosanilin dyes, but is less intense, more selective in its action and more subject to differentiation; third, in the vital staining of nervous tissue, where a nontoxic, basic dye is needed; fourth, in combination with eosin in the blood stains, thanks to the ease with which it can be partly converted into lower homologs such as the azures, and thus acquire polychrome properties; fifth, as an oxidation-reduction indicator, particularly in milk; and lastly, as an indicator in the Levine eosin-methylene-blue medium for differentiating the colon and aerogenes organisms.

It can be readily understood that an especially pure product is needed when the dye is to be used for vital staining or in blood work. For vital staining the U. S. P. zinc-free dye is always recommended, sometimes with even further purification; although the investigations carried on by the Commission indicate that the U. S. P. product is sufficiently pure. For blood work some companies sell a "methylene blue rectified for blood stains." This grade, however, is generally less pure than the medicinal or U. S. P. grade, and there seems no reason for specifying it. The same is true of various other grades such as those denoted BX, BG, etc., which are ordinarily purer than the textile dye, but less pure than the medicinal grade.

In former years, one or both of the German stain companies sold a grade designated "methylene blue for bacilli". This was often erroneously assumed to be specially adapted for staining bacteria. As a matter of fact, it proved to be the zinc salt, quite impure, and was probably labelled under the specified designation because it was not considered good enough for any other purpose! Even for staining bacteria it did not prove especially satisfactory. (It is, among other things, almost insoluble in alcohol, and cannot be used in any formula—such as that of Loeffler—which is derived from a stock alcoholic solution of the dye.) At present the American manufacturers, at least, have wisely discontinued the sale of the zinc salt under this designation. One American concern, however, still sells a "methylene blue for bacilli", but it is entirely different from the former imported product of that name. It is apparently methylene blue chloride, less pure than the medicinal grade but entirely satisfactory for staining bacteria and for many other staining purposes. The Commission has been unable, however, to find that it has any special advantages over the medicinal

product as a bacterial stain. It must be repeated that for all staining purposes, except possibly some lines of vital staining, medicinal methylene blue is an eminently satisfactory grade. When ordered for staining purposes it is best specified under the name: *methylene blue chloride.*

There is just one salt of methylene blue, other than the chloride, which is recommended by the Commission—namely *methylene blue thiocyanate.* This form of the dye, however, is not recommended as a stain but as an oxidation-reduction indicator for use in milk. Thornton and Sandin (1935) have shown that this salt has distinct advantages over the chloride in determining the rate of reduction of milk by its bacterial flora; the thiocyanate contains no water of hydration and hence can be obtained in almost 100% dye strength—a fact which makes for more ready standardization of solutions. Accordingly tablets of methylene blue for use in the reduction test in milk, which are put on the market in America are now wholly the thiocyanate.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Modified Wright stain for general tissue	IA_4-17
Tänzer-Unna orcein method for connective tissue	IB_3-16
Supravital staining of nerve endings (Ehrlich)	IC_3-34
Wright stain	ID_3-3
Tetrachrome stain, MacNeal	ID_3-5
Staining smears of bone marrow	ID_3-10-11
Staining sections of bone marrow	ID_3-11-13
Counterstain to alizarin red S for calcium deposits	ID_3-16
General staining of bacterial smears	$IIIA_2$-5
Counterstain in acid-fast staining	$IIIA_2$-8
Staining the diphtheria organism	$IIIA_2$-11
Macchiavello's stain for Rickettsiae	$IIIA_2$-19
Harris stain for Negri bodies	$IIIA_2$-20
Breed method for bacteria in milk smears	$IIIA_2$-21
Mallory's phloxine-methylene-blue stain	IA_4-20; $IIIB_3$-4
Lillie modification of Ziehl-Neelsen technic	$IIIB_3$-11
Kinyoun's diphtheria stain	$IIIA_2$-12
Stains for Negri bodies in sections	$IIIB_3$-12-13

Polychrome methylene blue. The oxidation of methylene blue into one or more of the compounds of lower methylation described on the preceding pages takes place in any methylene blue solution upon standing, especially rapidly if the solution be alkaline as in the Loeffler formula. All methylene blue solutions therefore, particularly if they have been standing any length of time, contain small amounts of the lower homologs, primarily azure A and azure B. These lower homologs are not only more

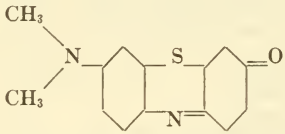
violet in color than methylene blue itself but are more selective in their staining action. It is indeed claimed (Scott and French, 1924b) that the dye owes much of its value as a nuclear stain to the azures that are present, and that an extremely pure methylene blue is not so satisfactory for such use. A later paper by Haynes (1927) indicates the correctness of such conclusions.

A methylene blue containing noticeably large proportions of the lower homologs is called *polychrome methylene blue.* The formation of these oxidation products may be hastened by boiling with alkali, as in Unna's formula.

Polychrome methylene blue is employed in many blood stains such as Leishmann's and Wright's; in the latter a methylene blue solution containing sodium bicarbonate is heated, and then eosin is added. Eosin enters into chemical combination with the basic dyes present, forming an insoluble compound which precipitates. This precipitate dissolved in methyl alcohol is Wright's stain. (For a more detailed discussion of the subject see Chapter XI).

j15 METHYLENE VIOLET (Bernthsen)

Methylene violet is formed whenever methylene blue is heated with a fixed alkali or alkali carbonate. It is a feeble base with the formula



$C_{14}H_{12}N_2OS$; *Mol. Wt.* 256.312

(*Absorption maximum 579–581*)

Its preparation from methylene blue is more difficult than that of azure A. A fair yield (30 to 40 per cent) may be obtained by oxidizing methylene blue in dilute ammoniacal solution with potassium chromate and then driving off the ammonia by boiling with the addition of sodium carbonate. It may also be prepared from azure A by boiling this with dilute alkali carbonate. Methylene violet precipitates out as needle crystals, insoluble in water. It may be recrystallized from dichlorethane ($CH_2Cl \cdot CH_2Cl$) in which it forms a deep carmine red solution. Although insoluble in water when pure, methylene violet is soluble when mixed with methylene blue or with the azures. It has a rather unusual shaped absorption curve, as shown in graph 3, Fig. 11, p. 105.

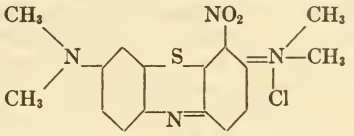
It plays an important part in the nuclear and granule staining of the polychrome methylene blue stains. A definite quantity of this dye is employed in the tetrachrome blood stain of MacNeal.

For formula and technic of **MacNeal's Tetrachrome Stain**, see Staining Procedures, p. ID_3-5.

For use as **counterstain to alizarin red S for calcium deposits**, see Id. p. ID_3-16.

Methylene violet (Bernthsen 1885) is not a textile dye and must not be confused with methylene violet RRA or 3RA, which is C. I. No. 842.

J20 METHYLENE GREEN C. I. NO. 924



$C_{16}H_{17}N_4O_2SCl$; *Mol. Wt.* 364.845

(*A basic dye; absorption maxima at about 660, 607.*)

(*Solubility at 26°C: in water 1.46%; in alcohol 0.12%*)

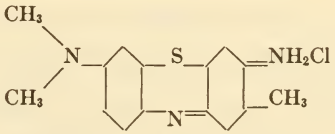
This dye is a mono-nitro methylene blue, obtained by the action of nitrous acid on methylene blue. The formula is probably as given above, but the exact position of the nitro group is uncertain.

It is occasionally used as a substitute for methyl green, especially by botanists in the case of wood and fixed chromatin, and gives good results in combination with eosin. It has also been employed by Lison (1938) as a vital stain for insects.

J25 TOLUIDINE BLUE O C. I. NO. 925

Synonym: *Methylene blue T 50 or T extra.*

This dye is closely related to thionin and to methylene blue in structure, and even more closely to azure A:



$C_{15}H_{16}N_3SCl$; *Mol. Wt.* 305.819

(*A basic dye; absorption maximum 620–622.*)

Solubility at 26°C: in water 3.82%; in alcohol 0.57%

In practice it proves difficult to distinguish this dye from azure A by spectrophotometric tests. This is particularly true because a shift in the absorption band of toluidine blue O sometimes occurs in

storage as a result of which the two dyes may become almost identical optically. Their great similarity is well brought out by comparing Curve 1 (toluidine blue) and Curve 2 (Azure A) in Fig. 12 on p. 108. Ball and Jackson (1953) have recently shown by chromatography that toluidine blue O seems to be a mixture, and suggest that this may account for much of the observed variation between samples.

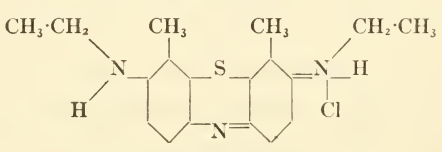
Although not a common textile dye, toluidine blue is more easily prepared than thionin or azure A—a fact of considerable importance, since in many procedures it may be substituted for one or the other of these dyes. It may be employed like azure A as a nuclear stain for sections of fixed tissue; and may be substituted for thionin in staining frozen sections of fresh tissue. It is a valuable general nuclear stain, being ordinarily employed in 0.3–1% aqueous solutions. It is widely employed in stains for Nissl granules and chromophylic bodies and has been proposed in a great variety of special procedures. Well known among the latter is Pappenheim's panchrome stain (of which it is an important ingredient) and the Albert stain which is at present widely replacing methylene blue in the diagnosis of diphtheria. It has use as a chemical reagent in the standardization of heparin and as a histochemical reagent in determining thymonucleic acid.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Staining frozen sections	IA_4-18-19 (note)
Bulk staining of Nissl granules	IC_3-15
Alizarin red S method of staining bone	ID_3-15
Albert's stain for diphtheria organism	$IIIA_2$-11
Kinyoun's stain for diphtheria organism	$IIIA_2$-12

J30 NEW METHYLENE BLUE N C. I. NO. 927

Synonym: *Methylene blue NN.*



$C_{18}H_{22}N_3SCl$; *Mol. Wt.* 347.897

(A basic dye; absorption maxima about [636.4], 588.)

Solubility at 26°C: in water 13.32%; in alcohol 1.65%

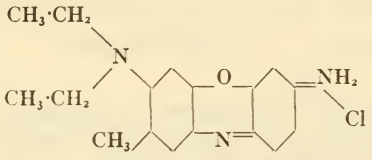
This dye has practically never been called for in microscopical work. The most interesting fact concerning it which has come to light relates to the Van Wijhe technic as applied by Louise Smith (1920) for staining the cartilage of frogs. The latter specified methylene blue, but the results could not be duplicated with any domestic or foreign methylene blue subsequently obtained. When furnished through the Commission with samples of various stains to try, she found that her earlier results could be duplicated with new methylene blue—a fact which not only implies mislabeling of her original supply of methylene blue, but suggests that new methylene blue may have some value in histological work.

4. THE OXAZINS

This group is like the thiazins in chemical formula except that the sulfur atom is replaced by an oxygen atom. Only a few of the dyes find use in microscopic technic, and they are not stains having very general application.

K5 BRILLIANT CRESYL BLUE C. I. NO. 877

Synonyms: *Cresyl blue 2RN* or *BBS. Brilliant blue C.*



$C_{17}H_{20}N_3OCl$; *Mol. Wt.* 317.811

(*A basic dye; absorption maxima about 624–628*)

This dye is expensive to manufacture, and as it is not at present employed in any line of commercial dyeing, it must be prepared specially for the biologist. Its manufacturers, although willing to coöperate to any reasonable extent, were naturally reluctant to carry on much experimental work with this dye unless they were sure the results would justify the outlay. This fact caused some delay in bringing about its complete standardization.

The spectral curve of a typical sample of brilliant cresyl blue is given in Fig. 13, p. 118, where it is compared with curves for resazurin and Nile blue sulfate.

The dye has been employed to some extent in vital staining, Irwin (1927), for instance, having found it the most favorable dye available for investigating penetration into living cells; in vital

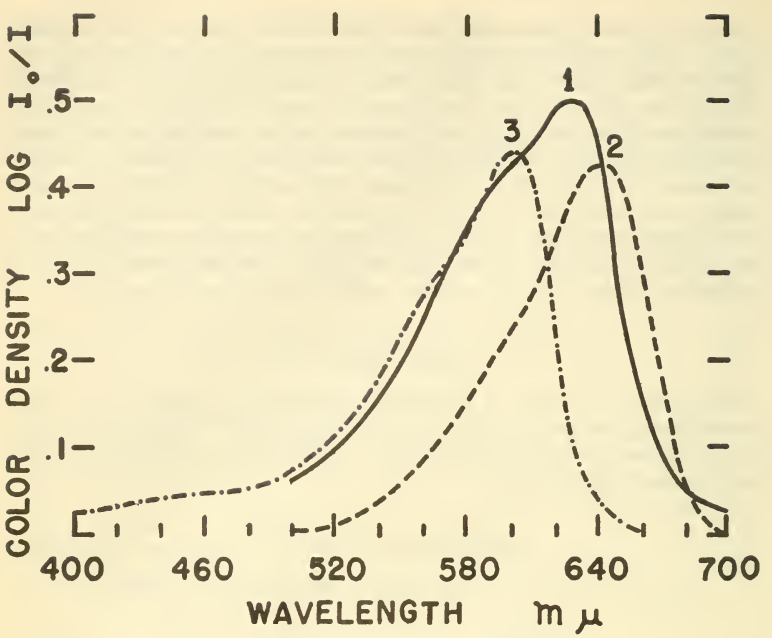


Fig. 13. Spectral curves of three oxazin dyes:
1. Brilliant cresyl blue. 2. Nile blue sulfate. 3. Resazurin.

staining it has special value because of its properties as an indicator of oxidation-reduction. Of recent years it has been employed for numerous purposes: staining protozoa, nematodes; in animal cytology; and as an amebicide. Its best known use, however, is as a blood stain to bring out reticulated cells and platelets. A well-tried procedure is that of Cunningham (1920), as modified by Isaacs (*in* McClung, 1937, p. 319); a somewhat different technic is that of Robertson (1917) for counting reticulated cells, and that of Buckman and Hallisey (1921) for platelets.

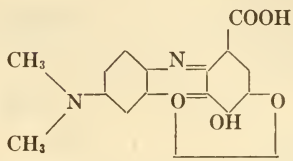
Of recent years some interest has been aroused by observations that certain derivatives of brilliant cresyl blue stain tumor cells selectively and retard the growth of tumors. See Lewis, Sloviter and Goland (1946). The relation between ability to stain and to retard growth is very interesting, even if not of practical significance.

For technic of **Cunningham's method of staining reticulated corpuscles and platelets** see Staining Procedures, p. ID_3-8.

For technic of **Robertsons' method for counting reticulated corpuscles**, see **Id.** p. ID_3-8.

K10 GALLOCYANIN C. I. NO. 883

Synonyms: *Alizarin blue RBN. Chrom blue GCB. Fast violet.*



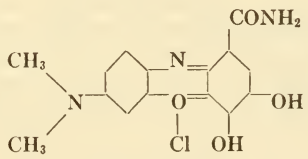
$C_{15}H_{12}N_2O_5$; *Mol. Wt.* 300.262

(*Absorption maximum about 636*)

Proescher and Arkush (1928) found the iron lake of this dye, prepared by boiling 2–3 minutes in a 5% aqueous solution of ferric ammonium sulfate, to be a splendid nuclear stain to be employed as a substitute for hematoxylin. Buzaglo (1934) used it, in combination with orcein and an acid alizarin blue, as a tissue stain. It has also found employment (Foley, 1943) as a counterstain in one of the protargol staining methods for nervous tissue.

For technic of **Foley's combined method for staining nerve fibers**, see Staining Procedures, p. IC_2-25.

K15 GALLAMIN BLUE C. I. NO. 894



$C_{15}H_{14}N_3O_4Cl$; *Mol. Wt.* 335.743

(*Absorption maximum about 651*)

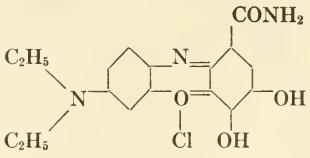
Employed in the form of its iron lake by Proescher and Arkush (1928) as a nuclear stain; the lake is prepared as described for gallocyanin above. It has been recommended by Stock (1949) for detecting calcium in tissues.

The following very similar dye has also been mentioned in biological literature, chiefly for its value as an oxidation-reduction indicator:

Prune pure; C. I. No. 893. Syn.: *Violet PDH. Gallo blue E.* Very similar to gallamin blue, but a methyl ester instead of an amide.

K20 CELESTIN BLUE B C. I. NO. 900

Synonym: *Coreine 2R.*



$C_{17}H_{18}N_3O_4Cl$; *Mol. Wt.* 363.795

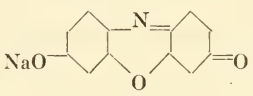
(*Absorption maxima about 654.5* [*600*])

Preferred by Proescher and Arkush (1928) to either gallocyanin or gallamin blue as a nuclear stain. As in the case of these two other dyes, the iron lake, prepared by boiling 2–3 minutes in a 5% aqueous solution of ferric ammonium sulfate, is employed for the staining solution. Lendrum and McFarlane (1940) recommend this dye in a modification of the Mallory connective tissue stain.

K25 LACMOID C. I. NO. 908

Synonyms: *Resorcin blue. Iris blue. Fluorescent blue.*

There is some confusion as to the actual chemical structure of this dye. It is recorded differently in different sources; and there is little question but that it varies according to the manufacturer from which it is obtained. At least one product of American manufacturer is believed to have the following structure:



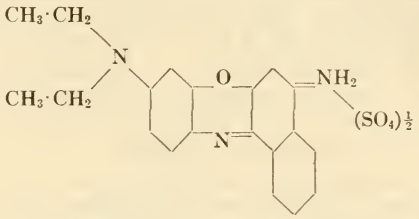
This does not agree with the Colour Index, under the number above cited, where a formula is given in which the NaO- radical is replaced by NH_4O-, and where a brominated derivative is regarded as properly called resorcin blue but not lacmoid.

Lacmoid has been used for its indicator properties. This dye, apparently, was employed by Tsvett (1911) under the name of resorcin blue, as a histochemical reagent for the detection of callose. Nebel more recently (1931) combined it with martius yellow (a dye at present unobtainable commercially) in a stain for pollen tubes.

K30 NILE BLUE SULFATE C. I. NO. 913

Synonym: *Nile blue A.*

There are two products sold at present under this name, one is a dimethyl aminophenol derivative, the other derived from diethyl aminophenol. The formula of the latter is:



$(C_{20}H_{20}N_3O)_2SO_4$; *Mol. Wt.* 732.828

(*A basic dye; absorption maxima 635–645*)

A spectral graph of brilliant cresyl blue, compared with resazurin and Nile blue sulfate, is given in Fig. 13, p. 118.

The use for which this dye is best known to the biologist is the Lorrain Smith fat stain (Smith and Mair, 1911). In this procedure the dye is boiled with dilute sulfuric acid, and thus hydrolyzed, with the introduction of oxygen in the place of the radical NH_2 $(SO_4)_{\frac{1}{2}}$, in other words producing a new dye of the class known as oxazones. This oxazone dye is red, and is fat-soluble. Nile blue sulfate itself, on the other hand, is not fat-soluble but combines readily with fatty acids. As a result the technic serves to distinguish between the free fatty acids in histological material and the neutralized fats, the former staining blue, the latter red. Knaysi (1941), however, calls attention to the fact that other dyes (e.g. neutral red or methylene blue) can be similarly employed.

Nile blue sulfate is used unaltered for staining living tadpoles previous to making transplants, in order to distinguish the grafts, also as a supravital stain for embryos (Detwiler, 1917) and as a vital stain for hydrae (Weimer, 1927), protozoa (Carter, 1933), yeasts (Gutstein, 1932) and amphibian eggs (Wintrebert, 1932). kland (1939) recommends it for staining bone sections, Tarao (1940) for histochemical studies of the Golgi apparatus. Foshay (1931) employs it for staining bacteria in tissues. The dye has been employed in culture media, containing emulsified fat, for the purpose of recognizing lipolytic bacteria; see Hammer and Collins (1934). Loosanoff (1937) finds it useful in marking starfish for identification.

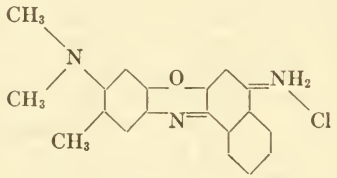
For technic of **Smith and Mair's stain for fats,** see Staining Procedures, p. ID_3-18.

K35 CRESYL VIOLET

CRESYLECHT VIOLET (i.e. CRESYL FAST VIOLET)

The exact chemical nature of imported cresyl violet is not yet known. Some samples appear to be mixtures of two dyes or possibly two different forms of the same dye. Samples from different sources frequently differ from one another but they are all closely related dyes. Spectral curves of two foreign samples are given in Fig. 14, graphs 2 & 3. They probably represent the type of dye with which biologists were familiar in the days before the world wars. Possibly, to distinguish them from the following, the name "cresyl fast violet" should be retained for this foreign product. It is not at present available.

At the present time a product of this name put on the market by the National Aniline and Chemical Co. seems to be different from any of the imported samples which have been examined. This National Aniline cresyl violet is better known chemically than the imported product and is considered to have the formula:



$C_{19}H_{18}N_3OCl$; *Mol. Wt.* 339.815

(*A basic dye; absorption maximum 624–630*)

Solubility at 26°C: in water 0.38%; in alcohol 0.25%

A spectral curve of this dye is also included in Fig. 14 (graph 1); a glance is sufficient to show how different it is from the foreign product.

Like brilliant cresyl blue, this dye is not employed on a commercial scale. Its chief value in histology is on account of its metachromatic properties. It has been employed in making permanent preparations of nervous tissue, and is excellent for staining fresh areolar connective tissue, as it brings out various histological elements. According to Ehrlich (1910, II, p. 78) it stains nuclei violet, plasma blue, amyloid, mucin and mast cell granules red. Spiridonovitch (1924) employs it in the vital staining of white blood cells. Williams (1923) uses it for staining sections of fresh tumor tissue in biopsy work. It is also employed for making permanent preparations of fixed tumor tissue. Landau (1934) proposes it for bulk staining of nervous tissue.

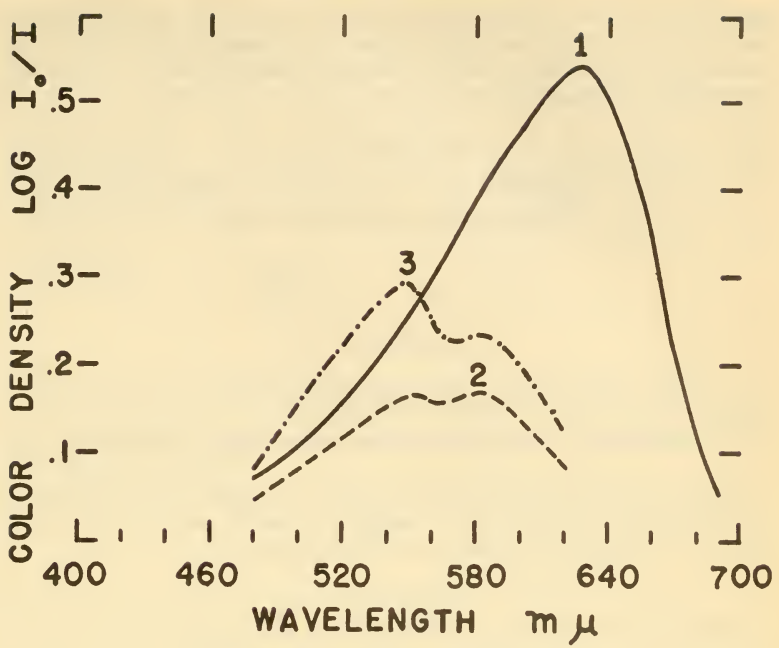


Fig. 14. Spectral curves of cresyl violet.

1. National Aniline product.

2. Kresylecht violet from Bayer; 3. from Grubler.

There is evidence that a stain satisfactory for one of these two latter purposes may not yield good results with the other. Williams, for example, finds rather better results with the National Aniline product in biopsy work, while from other quarters complaints have been received concerning the same batch of this dye when used for making preparations from fixed tumor tissue, for which the imported dye is very satisfactory.

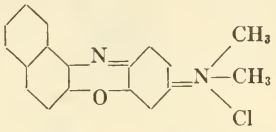
There have been conflicting reports about the relative merits of domestic and foreign products for neurological work. Quite unsatisfactory results have been reported with the National Aniline product as a stain for Nissl granules; but Banny and Clark (1950) find that a cresyl violet just obtained from Coleman and Bell is satisfactory. Their finding has not been verified by others. Furthermore, the latter product is not the same as the foreign samples shown by graphs 2 and 3 in Fig. 14. It is believed to be a diethyl (instead of dimethyl) amino compound.

It is plain that further work is necessary both on the chemistry of the imported product and on comparative value of the two types in staining. Experimental work on this dye has not pro-

gressed fast, both because of the small number of biologists who employ it and because of the expense to manufacture; but it is hoped that eventually it will be as well standardized as the more commonly used stains.

K40 NEW BLUE R C. I. NO. 909

Synonyms: *Naphthol blue R. Fast blue 3R. Phenylene blue. Meldola's blue. Indin blue 2RD.*

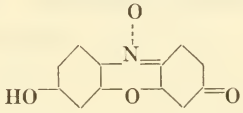


$C_{18}H_{15}N_2OCl$; *Mol. Wt.* 310.773

(*A basic dye; absorption maxima about* [*622*], *573*, [*533*])

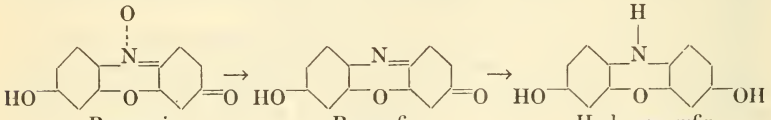
Occasional reference has been made to this dye in the biological literature, under the name of naphthol blue R, most recently by Lewis (1938) in staining fat drops in bacteria.

K45 RESAZURIN



$C_{12}H_7NO_4$; *Mol. Wt.* 229.184

This is not a dye, but a colored compound that has interesting properties as an oxidation-reduction indicator. It is blue in its completely oxidized state, and upon reduction becomes pink and then colorless according to the following reaction:



Various shades between blue and light pink can occur.

A spectral curve of resazurin is included in Fig. 13, p. 118 (graph 3).

It was first suggested by Ramsdell *et al.* (1935) that it might be more valuable than methylene blue as an indicator of reduction in milk, partly because of its greater speed in showing reduction and

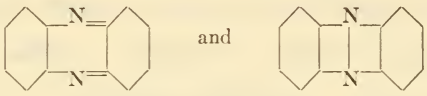
partly because of the value of the intermediate pink stage which does not occur with methylene blue. Its use was taken up quite promptly in Great Britain, but more slowly in the United States. It is now recognized as an alternate procedure by the Standard Methods of the Amer. Public Health Assn., and standardized tablets specially prepared for such use are on the market. For recent papers on the subject see Johns and Howson (1940) and Baker *et al.* (1942).

Another oxazin dye sometimes mentioned in connection with histology is:

Capri Blue. C. I. No. 876.

5. THE AZINS

The dyes of the azin group are derivatives of phenazin, $C_6H_4 \cdot N_2 \cdot C_6H_4$, a compound containing two benzene rings linked through two nitrogen atoms in such a way as to form a third ring. Two formulae are possible:

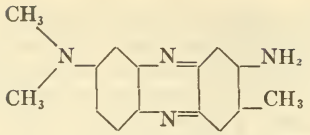


In the case of the first formula the quinoid ring is the chromophore; in the case of the second formula the azin group itself, $\begin{matrix}-N- \\ | \\ -N-\end{matrix}$, (see page 29) is assumed to be the chromophore. The quinoid formula is generally preferred today.

Phenazin is weakly basic, but is not a dye as it does not contain auxochrome groups. In other words, it is a chromogen. Either an $-OH$ group or one or more $-NH_2$ groups may be introduced to give it dye properties. The acids and bases are very weak if there is only one auxochrome group present, and their salts are readily decomposed. For this reason some of them are of use as indicators. Strong bases are encountered only among the safranins where basic character is derived not only from the two $-NH_2$ groups but also from one of the azin nitrogen atoms which becomes pentavalent and takes part in salt formation.

a. Amino-azins or Eurhodins

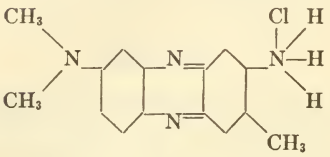
If one or more amino groups are introduced into a phenazin, a dye is formed of the class known as eurhodins. They are very weak bases, and therefore weak dyes; but as their salts are readily decomposed with a resulting color change, they form useful indicators. The best known of the group is toluylene red, base:



The chloride of toluylene red is the well-known neutral red.

L5 NEUTRAL RED C. I. NO. 825

Synonym: *Toluylene red.*



$C_{15}H_{17}N_4Cl$; *Mol. Wt.* 288.775

(*A weakly basic dye; absorption maximum 540–542*)

Solubility at 26°C: in water 5.64%; in alcohol 2.45%

This dye is yellow in solutions more basic than the neutral point (i.e., pH = 7.0) in reaction, and red in weak acids, even the reaction of ordinary tap water being sufficient to bring out the acid color; at a higher range of acid it turns blue. This gives it some value as an indicator. As an indicator it is also used in bacteriological media for distinguishing the colon from the typhoid organisms, and for recognizing other forms; although it is employed for this purpose much less today now that other dyes have been shown to have even greater value for the same type of work.

A spectral curve of this dye is included in Fig. 15, p. 127, together with one of azocarmine G.

Neutral red is employed in a variety of staining methods. It is a weak nuclear stain, and has value for that purpose in certain procedures. Twort's (1924) stain for parasites in tissues is a neutral stain (see Chapter VIII) formed by combining neutral red with light green and dissolving the precipitate in alcohol. Neutral red finds use in the study of the Golgi apparatus in cells; but there is a dispute as to whether it is actually a specific stain for this structure or merely for the inclusions of the Golgi apparatus. Old ripened solutions are employed for bringing out the Nissl granules in nerve cells. It also has some use in general histological staining, particularly for embryological tissue in combination with Janus green, as recommended by Faris (1924). Knaysi (1941) points out that it can be employed in place of Nile blue A in demonstrating hydrolysis of fats.

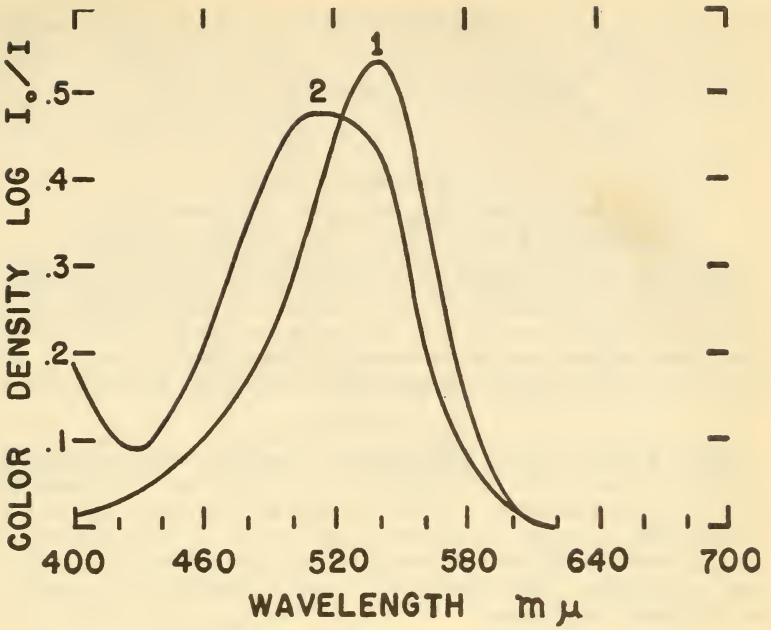
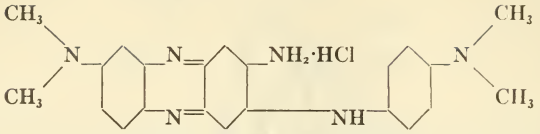


FIG. 15. Spectral curves of two azin dyes:
1. Neutral red. 2. Azocarmine G.

It has special value where a weakly basic, non-toxic dye is called for, as in vital staining. It is used for staining living protozoa, and as a vital stain for nuclei in tissue; also for the "vital" staining of blood, that is of fresh blood, observed under a microscope in a moist chamber, and for staining fresh gonorrhoeal pus under similar conditions. It proves useful as an indicator of reaction of the contents of living plant cells. The chief draw-back to neutral red in vital staining is the toxicity of certain lots that have been on the market. This toxicity seems to be due to impurities present in the dye. Phillips and Cohen (1927) have, in fact, shown that these impurities can be eliminated more readily by preparing the dye as an iodide instead of a chloride. Their process, however, has not been adopted by manufacturers as yet, because of technical difficulties. Nevertheless the recent samples of neutral red chloride submitted for certification have been so well purified that they seem to be free from toxicity.

For technic of **supravital staining of blood, with Janus green,** see Staining Procedures, p. ID_3-9.

L8 NEUTRAL VIOLET C. I. NO. 826



$C_{22}H_{25}N_6Cl$; *Mol. Wt.* 408.925

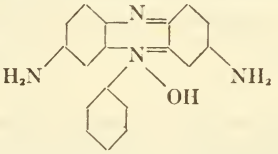
(*A weakly basic dye; absorption maximum 533*)

Solubility at 26°C: in water 3.27%; in alcohol 2.22%

This dye is very similar in its properties to neutral red, except that, due to its greater molecular weight, it is more bluish, giving a violet instead of a red color. It can be used as an indicator, but has been seldom used in histology. Unna (1921) however, has used it in a dye mixture employed in the study of chromolysis.

b. Safranins

Quite a long series of azin dyes are known in which one of the nitrogen atoms of the azin group is pentavalent and another benzene ring is attached to it. This pentavalent nitrogen allows the compounds to behave like ammonium bases; so with the amino groups which are always present, the basic properties of these dyes are very strong. The theoretical base of the simplest safranin would have the formula:

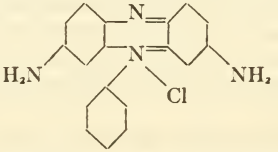


This form of ammonium base does not actually exist, as the safranin bases really occur in the form of anhydrides; but salts of these ammonium bases are the commonly known dyes. The commercial dyes are ordinarily chlorides.

There are two groups of safranins: the benzo-safranins in which the azin group unites two benzene rings; and the naphtho-safranins in which it unites two naphthalene groups. The simplest safranin is pheno-safranin, which is the chloride of the theoretical base just given, namely:

L15 PHENO-SAFRANIN C. I. NO. 840

Synonym: *Safranin B extra.*



$C_{18}H_{15}N_4Cl$; *Mol. Wt.* 322.789

Moore (1933) has called for phenosafranin in staining colonies of bacteria and fungi. The commercial safranins are ordinarily methyl or ethyl substitution products of this; or occasionally phenyl substitution products. The one of greatest value to the biologist is generally called safranin O.

L20 SAFRANIN O C. I. NO. 841

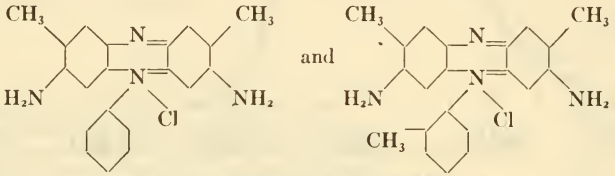
Synonyms: *Safranin Y or A. Gossypimine. Cotton red.*

Slightly different shades: *Safranin AG, T, MP,* and *G.* (Although all included in C. I. No. 841 they are different from the grade here described.)

(A basic dye; absorption maximum about 530)

Solubility at 26°C: in water 5.45%; in alcohol 3.41%

The common safranins of commerce, under various shade designations, are mixtures of di-methyl and tri-methyl phenosafranin:



$C_{20}H_{19}N_4Cl$; *Mol. Wt.* 350.841 $C_{21}H_{21}N_4Cl$; *Mol. Wt.* 364.867

The shade differs according to the proportion of these compounds present, the red being deeper according to the proportion of the tri-methyl compound in the mixture. The type safranin O, which proves best for ordinary biological purposes, can be defined as having its absorption maximum at 530mμ.

A spectral curve of safranin O is given in Fig. 16.

Safranin has always been a problem, because of its variability, especially in cytological work where it is often employed together with another basic dye, and a delicate balance between the two stains is necessary. The reason for this variation has been a puzzle ever since the Commission has begun its investigations of this dye. The question is not yet answered. It is realized at present, however, that there are samples that stain too blue, and others that stain too yellow, although this difference does not correlate with dye content nor with any chemical properties of the dye yet investigated. Strangest to say, there is no difference between any of these types of safranin that can be recognized by the spectrophotometer. Under such circumstances, no truly scientific method of standardizing the stain has been devised. An empirical method is in effect, however, which results in the present product

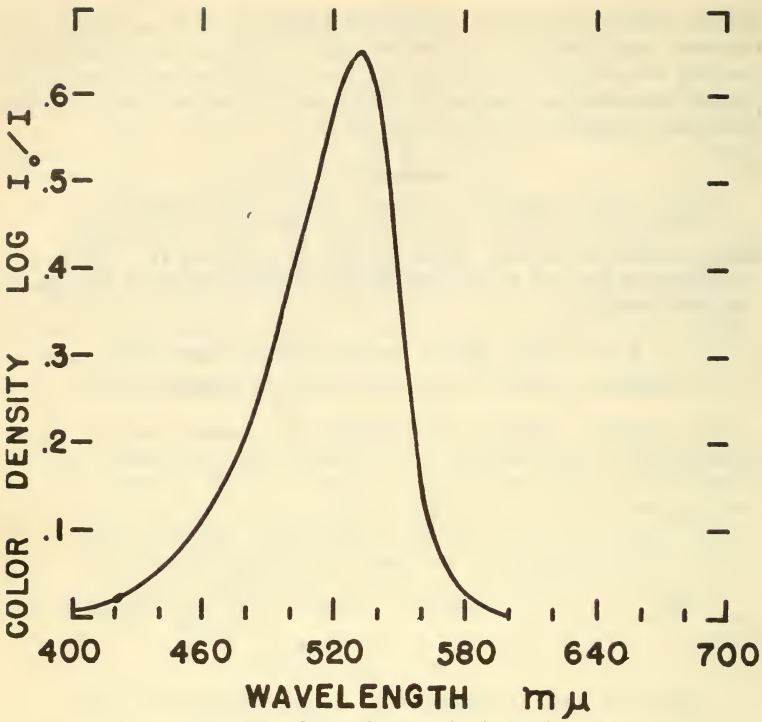


FIG. 16. Spectral curve of safranin O.

being distinctly better than that of former years. When a stain company submits a sample showing too blue or too yellow staining, the fault is called to the manufacturer's attention, and they try to submit another sample without that fault or with the opposite fault. This often means the submission of two batches, one too blue in its staining properties, the other too yellow; then it is a fairly simple matter to determine the proper mixture of the two to give best results. This has to be determined by actual staining tests, not by means of any colorimeter or spectrophotometer.

These observations undoubtedly explain the directions given by Chamberlain* to mix equal parts of alcohol soluble safranin and water soluble safranin for use in the Flemming triple stain. There is no such thing as a safranin insoluble in either alcohol or water; and the products formerly sold by Grübler as "wasserlöslich" and "spritlöslich" respectively have been found to be identical so far as chemical and optical analysis show. Probably, however, the particular batches formerly employed by Chamberlain were neither of them the correct shade in staining properties, while a

*In early editions of his Methods in Plant Histology.

mixture of the two gave good results. In other words he did about the same thing that the Commission now often recommends stain companies to do.

Grübler also sold other grades of safranin, none of which seem to have any special significance for biological purposes. One of these denoted "Safranin pur" was found on testing to be a more violet dye, apparently methylene violet (C. I. No. 842).

Safranin O is one of the most important nuclear stains known to the histologist. The botanist finds it especially valuable, as it brings out lignified and cutinized tissues in vascular plants, and can be employed in combination with a variety of contrast stains; it is valuable as a protein stain in plants, and can be used to stain spore coats. The cytologist makes use of it in the Benda technic to stain chromatin in combination with light green as a contrast stain; and even more widely in the Flemming triple stain, in which it is employed as a chromatin stain, together with gentian violet and orange G. The bacteriologist has some use for it, especially as a counterstain in the Gram technic.

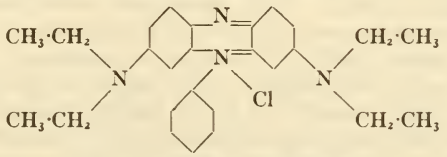
PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Counterstain to Weigert's iron hematoxylin	IA_4-6
Counterstain to $AgNO_3$ for calcium deposits	ID_3-17
Counterstain to iron alum hematoxylin in plant histology	IIA-9
With light green SF yellowish in plant histology	IIA-10
With picro-anilin-blue in plant histology	IIA-11
In Flemming's triple stain	IIB-10, 11
With fast green FCF in plant cytology	IIB-11
With anilin blue WS in plant cytology	IIB-12
Counterstain to Foster's tannic acid and $FeCl_3$	IIB-12
Counterstain in Gram technic, for smears	$IIIA_2$- 6
Schaeffer and Fulton spore stain for bacteria	$IIIA_2$-10
Counterstain in Gram technic, for sections	$IIIB_3$-9

L25 AMETHYST VIOLET C. I. NO. 847

Synonyms: *Heliotrope B. Iris violet.*

This dye is tetra-ethyl pheno-safranin:



$C_{26}H_{31}N_4Cl$; *Mol. Wt.* 434.997

(*A basic dye; absorption maxima about 589,* [*545.5*])

Solubility at 26°C: in water 3.12%; in alcohol 3.66%

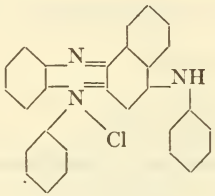
Amethyst violet has been used by Ehrlich and Lazarus (1898) as a basic dye in certain triple staining technics.

A further dye of this group which the biologist must take into account, although probably of no significance as a stain, is methylene violet RRA or 3RA, C. I. No. 842 (syn.: *fuchsia* or *safranin extra bluish*). This dye is a di-methyl safranin in which the methyl groups are introduced into one of the amino groups instead of directly into the benzene ring. It has no connection with the methylene violet of Bernthsen, which is one of the constituents of polychrome methylene blue; see p. 114. A sample has been found labelled "Safranin, pur", Grübler.

L30 AZOCARMINE G C. I. NO. 828

Synonyms: *Azocarmine GX. Rosazine. Rosinduline GXF.*

The sodium salt of a disulfonic acid of phenylrosinduline:



$C_{28}H_{20}N_3Cl$; *Mol. Wt.* 433.921

(*An acid dye; absorption maxima about 512*)

A spectral curve for this dye, together with one for neutral red, is given in Fig. 15, p. 127.

This dye is occasionally employed in tissue staining, notably after M. Heidenhain (1915) who used it as a tissue stain either alone or preceding some nuclear stain. Heidenhain gave special attention to its use in the Mallory anilin blue connective tissue stain; in which procedure he employed it in place of acid fuchsin in the Mallory technic. This azocarmine modification of the Mallory stain is very commonly followed according to the schedule given by McGregor (1929) which calls for staining in azocarmine, differentiation in anilin alcohol, then treatment in 5% phosphotungstic acid before application of the Mallory anilin-blue-orange-G mixture. (Unfortunately McGregor calls the dye "azan carmine".) Another modification is that of Volkmann and Strauss (1934) who used azocarmine after a resorcin gentian violet.

There is a closely related dye, azocarmine B (C. I. No. 829) which is a trisulfonate instead of a disulfonate. Heidenhain em-

ployed either, finding a stronger solution necessary in the case of azocarmine B, which required more application of heat (56°C.) in order to bring it into solution.

Besides the misspelling "azan carmine" one also frequently sees the name written as two words "azo carmine." The latter is not a serious mistake, but it is preferred here to write it as one word, so as not to imply that it is one of the azo dyes.

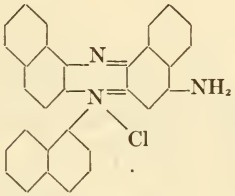
For technic of **Mallory-Heidenhain "azan" connective tissue stain,** see Staining Procedures, IB_3-4.

For technic of **Mollier's quadruple stain,** see Id. p. IB_3-7.

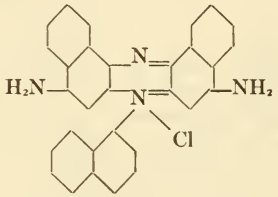
L35 MAGDALA RED C. I. NO. 857

Synonyms: *Naphthalene red. Naphthalene pink. Naphthylamine pink. Sudan red.*

This is a naphtho-safranin, and is a mixture of the monamino and diamino compounds:



and



$C_{30}H_{20}N_3Cl$; *Mol. Wt.* 457.941 $C_{30}H_{21}N_4Cl$; *Mol. Wt.* 472.957

(*A basic dye; absorption maximum about 524.*)

A true magdala red put on the market before 1914, and still sold by Grübler and Co., under the name of magdala rot *echt*, is quite expensive. This same company also sells a product "magdala rot der Handels," and a similar type has been sold by Coleman and Bell in this country. This so-called commercial magdala red is an entirely different dye, erythrosin or phloxine (see p. 195)–an acid instead of a basic dye, and one of an entirely different group. Chamberlain (1927) states that his work with magdala red was done with the latter type, hence actually with phloxine instead of the dye he thought he was using; his results cannot be duplicated with true magdala red. This means that in the well-known procedure for staining algae, using this dye in combination with anilin blue, one should actually employ phloxine, not magdala red. (See also discussion under phloxine, p. 196.) The same statement probably applies in the case of Dixon's (1920) magdala red and light green stain for mycelium in plant tissue.

A magdala red has been employed by Flemming as a nuclear

stain, and by Kultschitzky for staining elastic tissue. No information is available, however, as to whether they used phloxine or true magdala red.

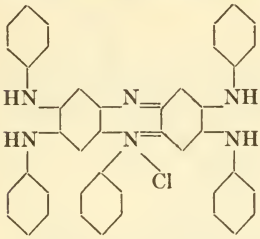
c. The Indulins

Indulins are similar to safranins but are more complex: being quite highly phenylated amino derivatives. Only a few concern us.

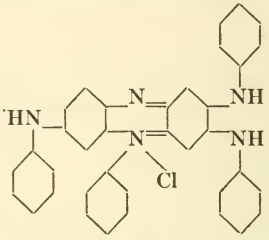
L40 **INDULIN, SPIRIT SOLUBLE** C. I. NO. 860

Synonyms: *Indulin* (with various shade designations). *Spirit indulin. Spirit nigrosin R.*

This consists of mixtures of the following:



and



$C_{42}H_{33}N_6Cl$; *Mol. Wt.* 657.189 $C_{36}H_{28}N_5Cl$; *Mol. Wt.* 566.081

(*A basic dye; absorption maximum varies from 541.5 to 560.5*)

L45 **INDULIN, WATER SOLUBLE** C. I. NO. 861

Synonyms: *Indulin* (with various shade designations). *Soluble indulin 3B. Fast blue B, OB, R, 2R,* etc.

(*An acid dye*)

This is the sodium salt of the sulfonation product of indulin, spirit soluble (C. I. No. 860). Because there are various mixtures of the latter and different degrees of sulfonation are possible, this dye may vary greatly in its composition and shade.

One of the indulins (exact nature uncertain) has been used by Lynch (1930) in the counterstaining of bulk tissue; also by Cumley (1935) in the negative staining of bacteria. For the latter purpose, however, the following dye is more commonly employed.

L50 NIGROSIN, WATER SOLUBLE C. I. NO. 865

Synonyms: *Nigrosin W, WL,* etc. *Gray R, B, BB. Silver gray. Steel gray. Indulin black.*

Nigrosin is not a pure dye, but is a mixture; and apparently the composition of different lots may vary. Ordinarily it is a mixture of a blue-black or violet indulin with a yellow dye in such a proportion that the resulting blend appears black. As the proportion of these two dyes is not always the same, different samples of nigrosin may vary in the amount of blue apparent to the eye. It is assumed that the biologist wants a nigrosin which appears distinctly black; this is certainly true in those procedures where it is used for a background stain. Accordingly the samples of nigrosin submitted which have a bluish hue have been refused certification by the Stain Commission.

Nigrosin is used in place of India ink as a background stain in the study of unstained bacteria; also as a stain for the background in contrast to fuchsin in the Dorner (1926) stain for bacterial spores. It is recommended by Ehrlich for staining the tissue of the central nervous system either alone or in combination with other stains, and by Jarotsky for staining pancreatic tissue following hematoxylin. Botanists use it in studying algae and fungi. Pfitzer's (1883) picronigrosin serves as a chromatin stain. Nigrosin is also used by Unna (1921) in combination with "orange" (orange G?) in the study of the process of chromolysis. Bean (1927) has employed it as a counterstain for nervous tissue.

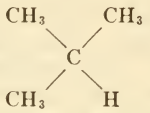
For the use in **negative staining of bacteria,** see Staining Procedures, p. $IIIA_2$-6.

For technic of **Dorner spore stain for bacteria,** see Id. p. $IIIA_2$-9.

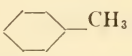
CHAPTER VII

THE PHENYL METHANE DYES

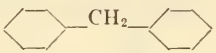
ONE of the most important groups of dyes, both from the standpoint of the dyer and from that of the biologist, is a group of substituted methanes, or in other words compounds with a central carbon atom. In methane, CH_4, it is possible to replace any of the hydrogen atoms with methyl, ethyl, or phenyl groups. If one H is replaced with CH_3, it becomes ethane, $CH_3 \cdot CH_3$. If two are replaced with CH_3 groups it becomes propane, $CH_3 \cdot CH_2 \cdot CH_3$; while if there are three substituent CH_3 groups it becomes iso-butane:



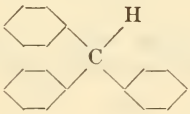
Similarly if one H is replaced with a phenyl group it becomes phenyl methane or toluene:



if with two it becomes di-phenyl methane:

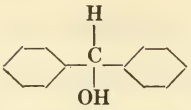


if with three it becomes tri-phenyl methane:



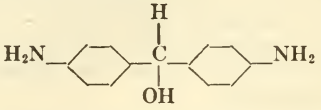
Certain substitution products of the di- and tri-phenyl methanes are among the most powerful dyes known.

Di- and tri-phenyl methane, themselves, are not dyes, nor are they chromogens. They lack both the chromophore and the auxochrome groups. The first step (theoretically) in converting them into dyes is to introduce an –OH group in the place of one of the unsubstituted H atoms of the methane nucleus. The compound thus formed, which bears the same relation to the phenyl methane as alcohol does to methane, is called a carbinol. A carbinol is methyl alcohol in which one or more of the hydrogen atoms may have been replaced with an alkyl radical or a benzene ring. Thus:

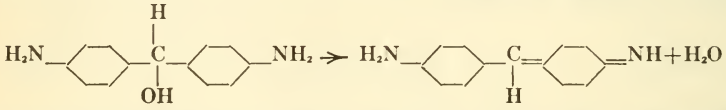


diphenyl carbinol

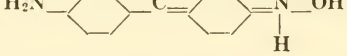
It is next theoretically possible to attach amino groups to the benzene rings. Thus in the case of di-phenyl carbinol it is possible to obtain di-amino di-phenyl carbinol:



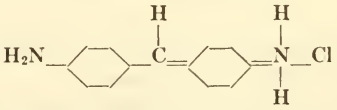
Now this latter compound contains the necessary auxochrome groups; but it is not yet a dye. No carbinol is a dye, because it lacks a chromophore group. The carbinols are important in dye chemistry, however, because upon dehydration a rearrangement of the bonds in the molecule takes place giving the quinoid benzene ring, which as we have seen is a powerful chromophore. Thus:



This latter compound is colored and is the anhydride of a true dye base. Upon hydration it should theoretically become:



Such a compound could exist only in aqueous solution. It is known only by its salts, the true dyes, as:



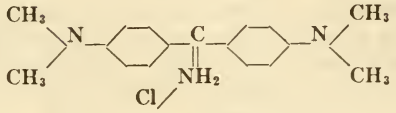
Although the theoretical compound given above is the true dye base, the carbinols are often known as carbinol bases or color bases of the phenyl methane dyes. As stated above, they lack the chromophore group, and hence are colorless.

1. DI-PHENYL METHANE DERIVATIVES

The di-phenyl methanes are seldom of biological significance. Only one deserves mention here.

M5 AURAMINE O C. I. NO. 655

Synonyms: *Canary yellow. Pyoktaninum aureum. Pyoktanin yellow.*



$C_{17}H_{22}N_3Cl$; *Mol. Wt.* 303.827

(A basic dye; absorption maxima [380], 431-434)

Solubility at 26°C: in water 0.74%; in alcohol 4.49%

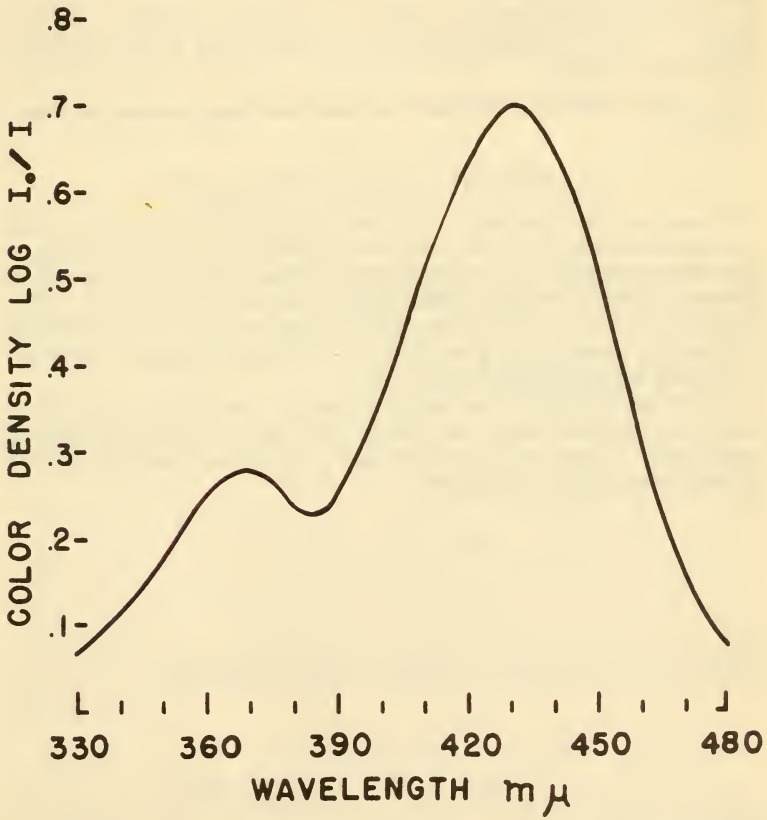


FIG. 17. Spectral curve of auramine O.

Auramine O has been in use for some time as a drug, but until recently has been regarded as having little value in microscopic technic. A search of the literature in 1936 revealed only the following rather obscure staining procedures calling for this dye: by Vinassa (1891) for staining plant sections; by Fischel (1901) for vital staining of salamander larvae; and by Kisser (1931), with cresyl violet as a tissue stain. More recently, however, its properties as a fluorochrome have brought it into considerable prominence in fluorescence microscopy. Richards and Miller (1941) recommend it as the best, among numerous fluorochromes investigated by them, for staining the tubercle organism. Their method, because of the low magnification and large fields which it makes possible, allows such an increase in the number of positives in diagnosis that it has attracted much attention to this dye since their method appeared.

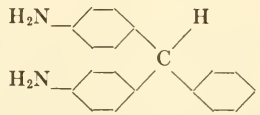
The spectrophotometric curve of a typical sample is given in Fig. 17. The most striking feature of this curve is the secondary maximum at 380.

For technic of fluorescent staining of acid-fast bacteria, see Staining Procedures,* p. $IIIA_2$-9.

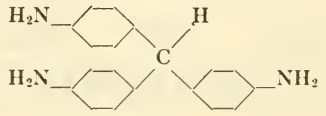
2. TRI-PHENYL METHANE DERIVATIVES

There are two groups of tri-phenyl methanes to concern us, the amino and the hydroxy derivatives. The former, which are much the more numerous, are very strongly basic, thanks to the amino groups, unless sulfonated like light green or acid fuchsin. The rosolic acid dyes, on the other hand, are hydroxy phenyl methanes, the amino groups being replaced by hydroxyl groups; they are therefore acid instead of basic dyes.

There are likewise two subdivisions of the amino derivatives, the di-amino tri-phenyl methanes and the tri-amino tri-phenyl methanes. These two groups are derivatives respectively of: di-amino tri-phenyl methane



and tri-amino tri-phenyl methane, or paraleucanilin.



*Conn and Darrow (1943-4).

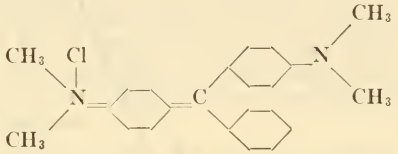
The individual dyes of this series are substitution products of these two compounds and differ from one another in the number of methyl, ethyl, or phenyl groups introduced, and according to whether they are introduced into the amino groups or directly onto the benzene rings.

a. Di-amino Tri-phenyl Methanes

M50 MALACHITE GREEN C. I. NO. 657

Synonyms: *Victoria green B or WB. New Victoria green extra, O, I or II. Diamond green B, BX, or P extra. Solid green O. Light green N.*

Various brands denoted: Malachite green A, B, BX, 4B, J3E, J3ES, NB, NH, or NJ.



$C_{23}H_{25}N_2Cl$; *Mol. Wt.* 364.903

(*Absorption maxima: 617–619,* [*425*])

See Fig. 18 for spectral curve.

Malachite green is a rather weakly basic dye that has been used in the past for various histological purposes; as by v. Beneden for staining Ascaris eggs, by Petroff for staining erythrocytes, and by Maas as a contrast stain following borax carmine; see Krause (1926-7), p. 1353. Today it has very largely been replaced by methyl green; but it is still called for in special procedures, of which the following might be listed by way of illustration: with acid fuchsin and martius yellow in the Pianese (1896) technic which was originally applied to cancer tissue but is now much more widely used in plant pathology; in its reduced (leuco) form, by Chapman and Lieb (1937), as a reagent (although less sensitive than crystal violet) for bacterial polysaccharides; as a bacteriostatic or amebicidic agent (Verona, 1935; Tsuchiya, 1936); as a vital stain for nulei of epidermis stains of the onion (Bank, 1938); by Schaeffer and Fulton (1933) as a bacterial spore stain; in place of methyl green in the Pappenheim stain, when combined with the Gram stain (Sandiford, 1938); also as a constituent of certain bacterial stains.

For technic of **Pianese IIIB stain for fungi in plant tissue,** see Staining Procedures, p. IIA-12.

For technic of **Schaeffer and Fulton method of spore staining,** see Id. p. $IIIA_2$-10.

For technic of **Laybourn modification of Albert stain for diphtheria** (which calls for this dye, mixed with toluidine blue), see Id. p. $IIIA_2$-11.

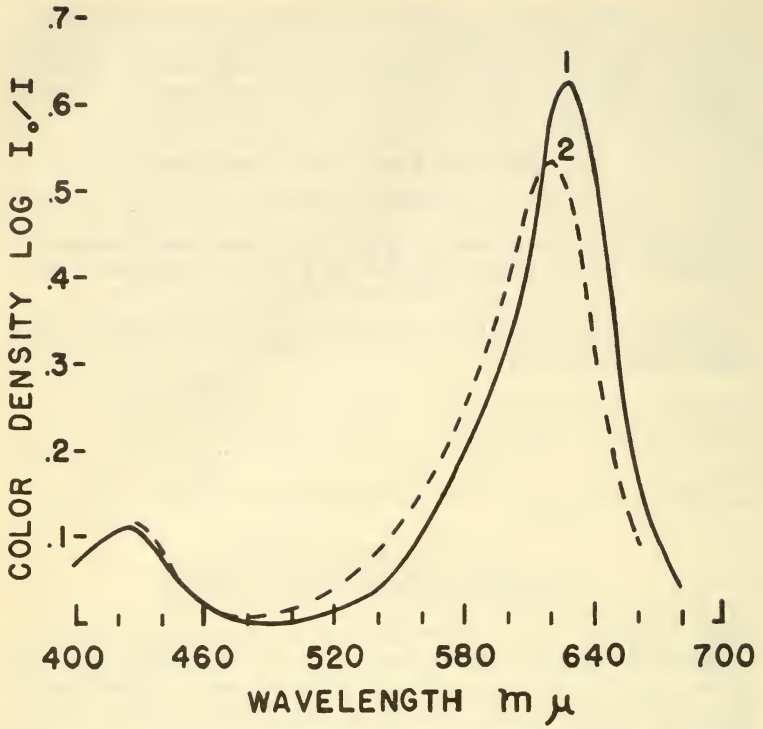
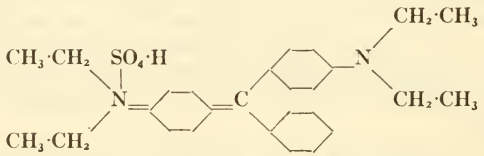


FIG. 18. Spectral curves of two phenyl methane dyes:
1. Brilliant green. 2. Malachite green.

M55 BRILLIANT GREEN C. I. NO. 662

Synonyms: *Ethyl green. Malachite green G. Emerald green crystals.** *Solid green JJO. Diamond green G.*

This is a basic dye which is generally known in the form of the acid sulfate:



$C_{27}H_{34}N_2O_4S$; *Mol. Wt.* 482.618

(*Absorption maximum: 628–631*, [*425*])

*Malachite green, also, has been exported from Germany under this name

Brilliant green is used to some extent as a stain for bacteria, spirochaetes, molds, and yeasts (see, for instance, Krajian, 1941), but finds more frequent employment as a constituent of bacteriological media. It is used, for example, as follows: (1) as a constituent of Krumwiede's brilliant green bile media for plating water in order to distinguish the colon organism from other lactose fermenting organisms; (2) it is employed in media used in searching for the typhoid organism in stools, where its value comes from its ability to inhibit the colon organism; (3) it is used as a constituent of enrichment media for the stimulation of the typhoid organism, in which it must prevent the growth of the normal colon types but not be toxic to the typhoid organism. It has proved much more simple to find a brilliant green satisfactory for colon diagnosis in water work than for the isolation and enrichment of the typhoid organism. A paper by Rakieten and Rettger (1927) has shown the difficulty encountered in this latter instance.

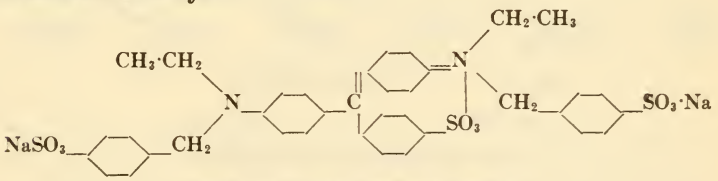
As the first of three purposes above mentioned is the one for which brilliant green is now most commonly used in America, all samples submitted for certification are tested for use in the brilliant green bile medium.

Spectrophotometric curves of typical samples of malachite green and brilliant green are given in Fig. 18. All green dyes have two absorption maxima, one about 430, the other about 620–630. The primary maximum for malachite green, however, is at a definitely shorter wavelength than that of brilliant green or either of the two following dyes.

M60 LIGHT GREEN SF YELLOWISH C. I. NO. 670

Synonyms: *Light green 2G, S or 2GN. Acid green* (with various shade designations). *Fast acid green N.*

This is a derivative of brilliant green, which is sulfonated and is therefore an acid dye.



$C_{37}H_{34}N_2O_9S_3Na_2$; *Mol. Wt.* 792.832

(*Absorption maximum: 629–634*, [*428*])

Solubility at 26°C: in water 20.35%; in alcohol 0.82%

Light green is a valuable plasma stain often used for staining animal tissues in contrast to iron hematoxylin or other nuclear dyes, although it fades badly if exposed to bright light. It is a

very valuable counterstain to safranin, especially after Flemming fixation, thus finding employment in cytological work. It photographs well. It is a constituent, together with neutral red, of the compound dye employed in the Twort (1924) stain for microörganisms in tissues and has been applied to the staining of bacteria, yeasts, algae, etc., under various conditions. In plant histology it is a useful cytoplasm and cellulose stain and has been employed by Buchholz (1931), mixed with acid fuchsin, for staining pollen tubes. Its greatest drawback is that it fades rapidly and is therefore not very permanent. Where greater permanency is desired the following dye may often be substituted for it.

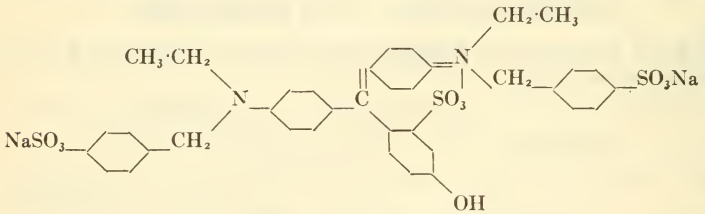
PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED*

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Crossman's modification of Mallory collagen stain	IB_3-5
Counterstain to Heidenhain's hematoxylin for plant tissue	IIA-7
Safranin and light green in plant histology	IIA-10
Counterstain to Feulgen stain for nucleoli in plant cytology	IIB-14

*Under this heading are given references to procedures described in detail in STAINING PROCEDURES, edited by Conn and Darrow (1943-4).

M65 FAST GREEN FCF

This is a dye, very closely related to light green SF yellowish, which was originally proposed by Johnson and Staub (1927) as a food dye.



$C_{37}H_{34}N_2O_{10}S_3Na_2$; *Mol. Wt.* 808.832

(*An acid dye; absorption maxima about 625,* [*420–430*])

Solubility at 26°C: in water 16.04%; in alcohol 0.35%

This dye was first tried in the Commission laboratory as a substitute for light green SF yellowish (see Haynes 1928) and has now come into general use in plant histology and cytology. It gives staining effects very much like light green and is consider-

ably less subject to fading. Slides have been exposed to direct sunlight for a few weeks and have still retained the green color. This dye is therefore to be recommended for such use as it has all the advantages of light green without the disadvantages. Considering the comparatively short period of time since this dye was first introduced to the microscopist, it is now employed in a surprisingly large number of procedures, among which might be mentioned the following as illustrating two widely different types of

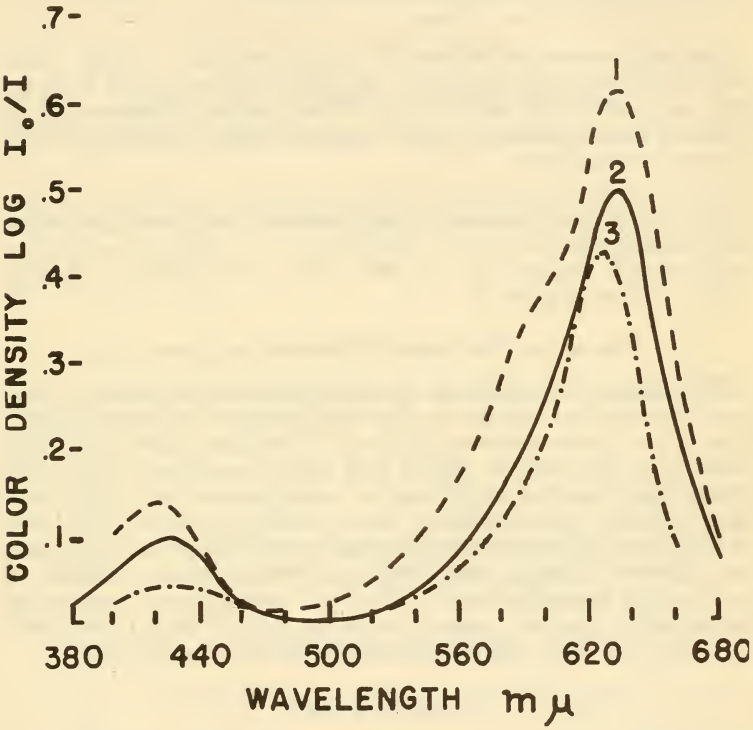


Fig. 19. Spectral curves of three phenyl methane dyes:
1. Methyl green. 2. Light green SFY. 3. Fast green FCF.

methods: the quadruple staining technic of Johansen (1939), for plant histology, in which it is combined with safranin, methyl violet, and orange G; its use as a bacterial stain by Maneval, (1941), who recommends this and other acid dyes (e.g. acid fuchsin, aniline blue W.S., and orseilline) as preferable to the conventional basic dyes because of the better differentiation afforded and the less tendency for slime and debris to take the stain.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Kornhauser's "Quad" stain for tissue	IB_3-8
Modification of Van Gieson stain	IB_3-11
Lillie modification of Masson trichrome stain	IB_3-19
Safranin and fast green in plant cytology	IIB-11
De Tomasi modification of Feulgen stain in plant cytology	IIB-13

Spectrophotometric curves of typical samples of light green and fast green are given in Fig. 19. It will be seen that they are both very much like the curve for brilliant green, although slightly different from malachite green in the location of the primary absorption maximum.

Another dye of this group which has been mentioned in biological literature is:

Acid green L Extra; C. I. No. 666. Syn.: *Acid green G, B extra, GV. Pontacyl green B.*

b. Tri-amino Tri-phenyl Methanes (Rosanilins)

The simplest rosanilins are the dyes sold as basic fuchsin. This term is somewhat loosely used to apply to two or three different dyes or to various mixtures of them. The dyes known as fuchsin differ from the methyl violets and other rosanilins in that the amino groups are not methylated or substituted in any other way. The fuchsins may, however, have methyl groups introduced directly onto the benzene rings instead of into the amino groups; and the different fuchsins vary from one another in the number of such methyl groups present. There are four primary compounds theoretically possible, namely with no methyl group, and with one, two, and three substituent methyl groups respectively.

BASIC FUCHSIN

Synonyms: *Fuchsin RFN. Magenta. Basic rubin. Anilin red.**

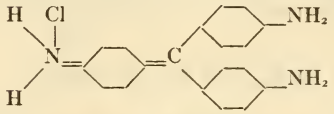
The dyes ordinarily known to commerce as basic fuchsin are represented by C. I. No. 677, which is a mixture of pararosanilin, rosanilin and magenta II. Pararosanilin is obtainable in fairly pure form, as is also the compound with three substituent methyl groups, namely new fuchsin. The formulae of these compounds follow:

*An impure basic fuchsin known as *cerise* has been mentioned in the biological literature.

N2 Pararosanilin (Magenta O) C. I. NO. 676

Synonyms: *Basic rubin. Parafuchsin. Paramagenta.*

This compound is triamino-triphenyl-methane chloride.



$C_{19}H_{18}N_3Cl$; *Mol. Wt.* 323.815

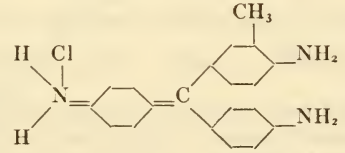
(*A basic dye; absorption maximum about 545*)

Solubility at 26°C: in water 0.26%; in alcohol 5.93%

It is the chief constituent of the majority of samples of basic fuchsin submitted for certification as biological stains. It is sometimes furnished as the chloride, sometimes as the acetate, generally the latter.

N3 Rosanilin (Magenta I)

This compound is mono-methyl fuchsin, or triamino-tolyl-diphenyl-methane chloride.



$C_{20}H_{20}N_3Cl$; *Mol. Wt.* 337.841

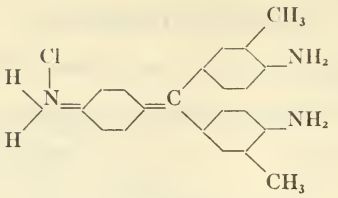
(*A basic dye; absorption maximum about 550*)

Solubility at 26°: in water 0.39%; in alcohol 8.16%

It is not a textile dye, and is not found free from pararosanilin unless specially prepared.

N4 Magenta II

This theoretical constituent of basic fuchsin is never encountered pure except as specially prepared in the laboratory, but probably is present in market samples of the dye. It is di-methyl fuchsin, or triamino-ditolyl-phenyl-methane chloride.



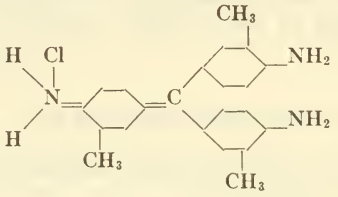
$C_{21}H_{22}N_3Cl$; *Mol. Wt.* 351.867

(*A basic dye; absorption maximum about 554*)

N5 New Fuchsin (Magenta III) C. I. NO. 678

Synonyms: *Isorubin. Fuchsin NB.*

This compound is tri-methyl fuchsin, or triamino-tritolyl-methane chloride:



$C_{22}H_{24}N_3Cl$; *Mol. Wt.* 365.893

(*A basic dye; absorption maximum about 556*)

Solubility at 26°C: in water 1.13%; in alcohol 3.20%

From the general rule concerning alkyl substitution it will be understood that new fuchsin is the deepest in shade of these four dyes, pararosanilin the least so. The commercial fuchsins, therefore, which consist of mixtures of the first three vary in depth according to the proportions in which they are present.

A typical spectrophotometric curve of rosanilin (Magenta I) is given in Fig. 20. Those for Magentas O, II, and III are not given here, as they are so nearly the same that they can be distinguished only by careful measurement, and the differences would not show in such a graph as those here included.

It has proved from the investigations of the Stain Commission that not all basic fuchsins furnished as biological stains are mixtures of these three compounds. Many of the samples prove to be fairly pure pararosanilin; while others are deeper in shade than rosanilin and presumably contain appreciable quantities of magenta II. All of these dyes differ so very slightly from each

other in hue that it takes very careful comparison to distinguish one from another, and for many purposes they may be used interchangeably.

The basic fuchsins are among the most powerful nuclear dyes, and find many biological uses. They are valuable stains for mucin, for elastic tissue, and for bringing out the so-called fuchsinophile

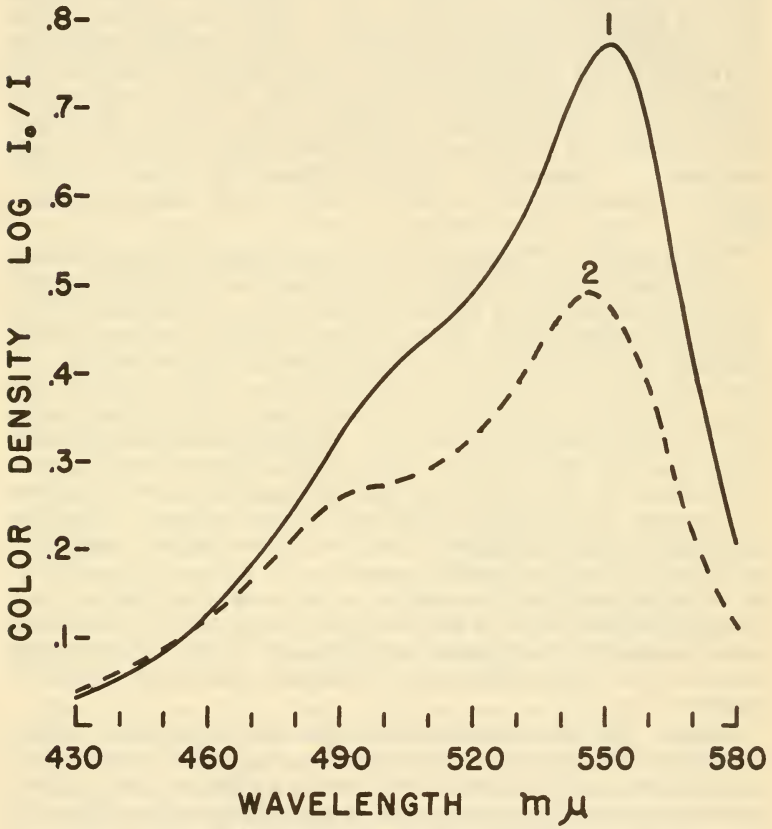


Fig. 20. Spectral curves of two rosanilins:
1. Basic fuchsin. 2. Acid fuchsin.

granules. A basic fuchsin is often employed for staining the nuclear elements of the central nervous tissue. This group of dyes is almost indispensable in bacteriology, particularly in the Ziehl-Neelsen method, with its various modifications, for differentiating the tubercle organism and thus making possible the diagnosis of tuberculosis.

Basic fuchsin is also employed as a chemical reagent in a determination which is coming to be of considerable interest to the biologist. Basic fuchsin is the main constituent of Schiff's reagent, often employed for the detection of aldehydes, although not an absolutely specific reagent for the purpose. In preparing this reagent, basic fuchsin is reduced through the action of sulfite to a colorless form, having the type of the formula for leuco-fuchsin which is given at the middle of page 31. (The formula there given for leuco-fuchsin, however, is not exactly that of Schiff's reagent, as it is believed that the sulfite radical in some way enters into its composition.) In the presence of aldehyde some chemical reaction not wholly understood takes place which evidently restores the quinonoid structure of the molecule and, accordingly, the color of the compound. Apparently a slightly different dye from the basic fuchsin is thus produced, since the color is violet rather than pure red.

This reaction is of interest to the bacteriologist, as basic fuchsin is employed similarly in the Endo medium for distinguishing between members of the colon-typhoid group of bacteria. This medium contains lactose, which is not acted upon by the typhoid organism but is fermented by organisms like *Bacterium coli*. It has been known for many years that these lactose fermenting organisms of the group restore the red color of the medium, while the non-lactose-fermenters do not. The suggestion was naturally made that this Endo reaction might depend upon the production of aldehyde by the organisms, but for years no one was able to demonstrate the presence of aldehyde in such cultures. Neuberg and Nord (1919) suggested, however, that aldehyde might be produced but not ordinarily accumulate in sufficient quantity to be detected; and that the sulfite in the medium might act as a "trapping agent" so as to allow it to accumulate. This has since been verified by Margolena and Hansen (1933). Apparently, therefore, the Endo medium is a biological application of Schiff's reagent.

Feulgen and his associates (Feulgen and Rossenbeck, 1924; Feulgen and Voit, 1924) employed this compound of basic fuchsin and sulfurous acid in histological technic, regarding it as a microchemical reagent for detecting the presence of aldehyde-like substances in the nuclei. Feulgen called this the "nucleal reaction," the ending "-al" indicating the aldehyde-like nature of the substance which he considered the reaction to demonstrate. This interpretation of the Feulgen reaction is now regarded as only part of the story; the significance of the desoxyribosenucleic acid demonstrated by the method is appreciated more today than it was when Feulgen proposed the method.

As a matter of fact, the Schiff reagent, employed by some modification of the Feulgen technic, has given histochemistry its

greatest impetus. Special significance has attached to the HIO_4-Schiff technic, in which the use of the Schiff reagent is preceded by oxidation with periodic acid (HIO_4), as first proposed by McManus (1946). It is considered that certain polysaccharides (and their derivatives) which contain 1,2-glycol and α-amino-alcohol groups react with the periodic acid (or other oxidant) to produce aldehyde groups, and that the latter react with the Schiff reagent to bring out the violet color. This has enabled histochemists to determine the locations of the compounds in question within the cell. Histochemists are proving much more exacting in their requirements for a basic fuchsin than were histologists or chemists using the Schiff reagent. As a result of trying to meet their requirements, the quality of basic fuchsin on the American market has been improving in recent years.

Early editions of this book contained statements, realized at the time to be largely provisional, to indicate which types of fuchsin are most satisfactory for the various purposes. Data that have been accumulated since 1929, however, seem to show pretty definitely that the exact chemical nature of basic fuchsin itself is of comparatively little importance. Good results for practically any purpose have been obtained with pararosanilin, rosanilin, new fuchsin, and even with the seldom-encountered magenta II; and the dyes have proved equally satisfactory whether in the form of chlorides or acetates, the two most common salts of this dye. From the standpoint of ordinary staining, in fact, it was realized a long while ago that the samples furnished by the stain companies varied very little in their performance.

In their behavior in the Endo medium and in the Feulgen technic, however, the samples were found to be quite dissimilar, and it was some time before any clue could be obtained as to the cause of their differences. It has finally been concluded, however, that the variations which occur are due to foreign ingredients which are almost always present in these dyes.

The problem of the Endo medium was solved first. It was learned (Conn and Darrow, 1934) that almost any sample of basic fuchsin will give good results in the Endo medium if the formula of that medium is carefully adjusted so as to avoid either an excess or a deficiency of sulfite in proportion to the fuchsin. Another complication arises from the fact that the 1936 edition of the Standard Methods of Water Analysis (of the American Public Health Association) lists two different formulae for the Endo medium, of which Formula II calls for K_2HPO_4, while formula I does not. For some reason, not yet explained, it is possible for a particular sample of basic fuchsin to give good results in Formula II while appearing too deeply colored in Formula I; or to be satisfactory in Formula I but not allowing sufficient color restoration by lactose fermenters if Formula II be used. As a result it is

necessary to select between these two formulae the one best suited to the particular batch of fuchsin to be employed. It is also well recognized today that many basic fuchsins contain an impurity which does not completely decolorize under the action of sulfite, and which becomes evident in such a reduced solution by the yellowish, or even brownish pigment remaining. The nature of this pigment has not been learned, altho Scanlan and Melin (1937) report an investigation of the subject. Following the publication of Scanlan and Melin's paper, the stain companies have begun to find ways of preparing fuchsins that are free from the undesirable impurity; and batches submitted for certification since then have ordinarily proved satisfactory for the Feulgen technic as well as for the other purposes discussed above. Not long after the above paper appeared Coleman (1938) reported a method of eliminating this impurity from a decolorized fuchsin by agitation with a decolorizing carbon; his method seems to make a sample of this dye usable in the Feulgen technic, even though previously entirely unsatisfactory. The histochemists, however, want a glass-clear Schiff reagent; and modern producers of stains in America are doing their best to supply basic fuchsins that will suit. This is at present one of the tests applied to samples submitted for certification. Lillie (1951) has contributed considerable information on the subject.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS DYE IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
HIO_4 leucofuchsin method for glycogen	IA-15
Weigert's resorcin fuchsin	IB_3-13
Lillie modification of Gallego elastic tissue stain	IB_3-15
Bauer's leucofuchsin method for glycogen	ID_3-22
Aceto-carmine and basic fuchsin for pollen tubes in style	IIA-13
DeTomasi modification of Feulgen stain in plant cytology	IIB-13
Feulgen stain for nucleoli in plant cytology	IIB-14
General staining of pure cultures of bacteria	$IIIA_2$-5
Ziehl-Neelsen stain for acid fast bacteria	$IIIA_2$-8
Dorner's spore stain for bacteria	$IIIA_2$-9
Snyder modification	$IIIA_2$-10
Casares-Gil's method for bacterial flagella	$IIIA_2$-14
Gray's method for bacterial flagella	$IIIA_2$-15
Liefson's method for bacterial flagella	$IIIA_2$-15
Bailey's method for bacterial flagella	$IIIA_2$-16
Hiss' method for bacterial capsules	$IIIA_2$-17
Macchiavello's stain for Rickettsiae	$IIIA_2$-19
Weigert's stain for fibrin and bacteria in tissues	$IIIB_3$-9
Verhoeff's carbol fuchsin for tubercle bacilli in tissues	$IIIB_3$-10
Lillie modification of Ziehl-Neelsen technic	$IIIB_3$-11
MacCallum's stain for influenza bacilli in tissues	$IIIB_3$-14
Williams' stain for Negri bodies	$IIIB_3$-13
Goodpasture's stain for influenza bacilli in tissues	$IIIB_3$-15

One comparatively recent complaint concerning certain samples of basic fuchsin relates to their behavior in staining tubercle bacteria by the acid-fast technic. It has been claimed that with certain samples of the dye these organisms show a tendency to decolorize partially or wholly, thus resulting in poorly stained cells that tend to appear as a string of beads. This has proved a difficult complaint to investigate, because consistent results are not obtained by different investigators. In fact, Yegian and Porter (1944) have shown that a normally staining culture may be converted into one which is not acid-fast or which shows beaded staining by improper methods of smearing on the slide. This observation makes it appear doubtful whether the complaints in question may or may not be due to variations between the different lots of fuchsin.

In general it can be stated, that in spite of all variations reported, the standardization of this dye seems to have been accomplished in a practical way, even if there is still much to be learned about it from a theoretical standpoint.

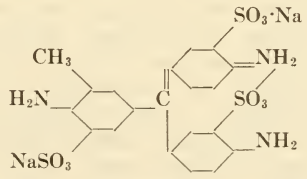
N12 ACID FUCHSIN C. I. NO. 692

Synonyms: *Fuchsin S, SN, SS, ST,* or *S III. Acid magenta. Acid rubin.*

(*An acid dye; absorption maximum: pararosanilin 540–543, rosanilin 543–546*)

This dye owes its acid character to the fact that it is a sulfonated derivative of basic fuchsin. Acid fuchsins are ordinarily rather complex mixtures. As there are four primary basic fuchsins possible, according to the degree of methyl substitution, and as each may yield at least three different compounds on sulfonation, fully a dozen acid fuchsins are theoretically possible, and samples are hardly to be expected which are not mixtures of several.

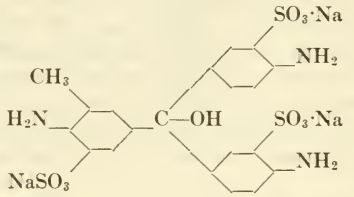
The generally accepted formula of one of the homologs present in acid fuchsin, namely the di-sodium salt of rosanilin trisulphonic acid, is:



$C_{20}H_{17}N_3O_9S_3Na_2$; *Mol. Wt.* 585.534

The bond connecting one of the sulfonic groups with an amino group attached to a different benzene ring is assumed to exist in

order to account for the fact that although only two of the sulfonic groups are neutralized with sodium, the compound acts as though it has no free acid. In other words, it is a case of intramolecular salt formation. Now when the tri-sodium salt is formed, this bond is broken down, whereupon the quinoid ring disappears and the following compound is produced:



This compound, it will be seen, is a carbinol in structure, and as it lacks the quinoid ring it is colorless; but it is very readily converted into the di-sodium salt by the addition of acid, whereupon the color again appears. This property makes acid fuchsin of use as an indicator. The decolorized solution of acid fuchsin neutralized with sodium hydroxide is called the Andrade indicator. It was once used extensively in bacteriological work, because of the striking reaction when its color is restored by acid-forming bacteria. (See Andrade-Penny, 1895.) As an indicator to show hydrogen-ion concentration at all accurately, however, it is found to have much less value than the phthalein and sulphonphthalein dyes (see pp. 197 to 206).

Acid fuchsin is a widely used plasma stain, which has also been recommended for a number of special uses. Among the best known are: the Van Gieson (1889) connective tissue stain, in which it is used with picric acid after hematoxylin to differentiate smooth muscle from connective tissue; the Ehrlich-Biondi tri-acid stain for blood, which is a "neutral" combination with orange G and methyl green (see Krause, 1926-7; pp. 457, 1707). In plant histology it is used to stain the cortex, pith and cellulose walls: while the Pianese (1896) stain (with malachite green and martius yellow), which was originally applied to cancer tissue, is now used by plant pathologists in studying infected vascular plants. It is used with methyl green, by Altmann, Bensley and Cowdry as a stain for mitochondria (see Lee, 1937, pp. 167-8). To the pathologist it is quite valuable as a constituent (with anilin blue and orange G) of the Mallory (1900) connective tissue stain.

Scanlan, French and Holmes, (1927) showed that many lots of acid fuchsin are unsatisfactory because of the rapidity with which they fade in Van Gieson preparations. These writers found that this fault arises from lack of control of the process of sulfonation. Their results indicated that a satisfactory product could be obtained if this process were carefully controlled by the use of a low

temperature (85°C.), fuming sulfuric acid containing sufficient free SO_3 to insure complete sulfonation to the trisulfonic acid, and careful acidification to yield only the disodium salt. Since the method which they proposed in this article was called to the attention of manufacturers, the American product has improved; it not only gives permanent Van Gieson preparations, but proves more satisfactory in the Mallory connective tissue stain.

The spectrophotometric curve of a typical acid magenta I (rosanilin) is given in Fig. 20 (curve 2), p. 149. The chief difference between this and the corresponding basic fuchsin is that it has a distinct secondary maximum at about 500, where the curve for basic rosanilin shows merely an almost imperceptible bulge. A dye having its primary maximum at 500 would be orange in shade, which is approximately the color of the acid phase of the Andrade indicator.

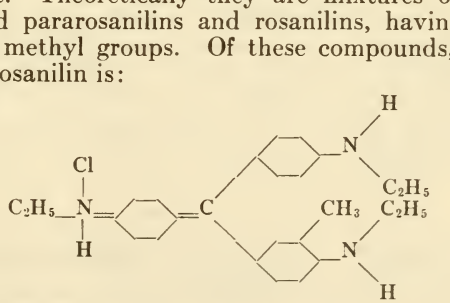
PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Van Gieson stain with iron hematoxylin	IA_4-21
Mallory's anilin blue collagen stain	IB_3-3
Crossman's modification	IB_3-5
Acid alizarin blue modification	IB_3-6
Masson's trichrome stain	IB_3-18
Pianese IIIB stain for fungi in plant tissue	IIA-12

N20 HOFMANN'S VIOLET C. I. NO. 679

Synonyms: *Dahlia. Iodine violet. Red violet. Violet R, RR or 4RN. Primula R water soluble.*

These various names are applied in a somewhat indiscriminate way to dyes intermediate in shade between basic fuchsin and methyl violet. Theoretically they are mixtures of methylated and ethylated pararosanilins and rosanilins, having fewer than five ethyl or methyl groups. Of these compounds, the formula for tri-ethyl rosanilin is:



$C_{26}H_{32}N_3Cl$; *Mol. Wt.* 421.997

(*A basic dye*)

In actual practice, however, the names applied to this group of dyes are very loosely employed, and one is often furnished under such a label as dahlia or Hofmann's violet a mixture of basic fuchsin and methyl violet, having a shade about the same as one of the compounds just mentioned. Two samples of "dahlia" have been called to the attention of the Commission which actually differed very greatly, one being nearly as red as fuchsin, the other nearly as blue as methyl violet. There was every reason to believe that one was a fuchsin with a small addition of methyl violet, the other a methyl violet with a little fuchsin added. The latter dyes are much more cheaply prepared than the true Hofmann violets, and the substitution is natural considering that the shade obtained can be the same.

Hofmann's violet has been called for by Ehrlich and by Unna for staining mast cells; by Juergens for staining amyloid, which it colors red, while the cytoplasm is colored blue. (See Krause, 1926-7, p. 1123). Now it is possible that for one of these procedures or for some other similar one, a true Hofmann's violet is necessary; on the other hand the shade obtained may be the important matter, in which case as satisfactory results should be obtainable with a mixture of fuchsin and methyl violet. This is a matter that should be further investigated; for if the cheaper fuchsins and methyl violets can be substituted for the Hofmann violets, the substitution can be made by the biologist himself. It is interesting to remark that one supply house not long ago sent out a bottle of crystal violet, unintentionally mislabeled dahlia, to a biologist ordering the latter dye. This biologist shortly wrote to the company that he would like more of it, as it was the best lot of dahlia he had ever used; in looking the matter up to duplicate the former order, the company discovered its mistake.

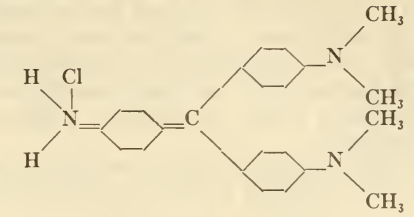
It is extremely difficult to determine just what dye in this group is referred to by any given name. Thus a rosanilin violet has been mentioned as having been employed in a stain for Negri bodies; and it is uncertain whether it is a dye of the above group or of the group following. Any biologist employing one of the violet rosanilins should be especially careful in publishing his work to give all the information furnished on the label as to manufacturer and the nature of the dye; and should preferably verify all such work, before publication, by using a dye of known composition.

N25 METHYL VIOLET C. I. NO. 680

Synonyms: *Dahlia B. Paris violet. Pyoktaninum coeruleum. Gentian violet.*

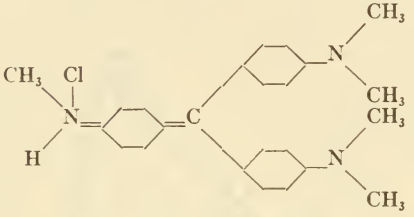
Various shades denoted: Methyl violet 2R, R, B, 2B, 3B, BBN, BO, V3.

The various dyes denoted methyl violet are mixtures of tetra-, penta-, and hexa-methyl pararosanilin:



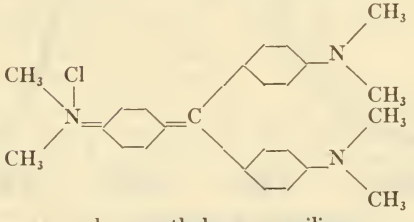
tetra-methyl pararosanilin

$C_{23}H_{26}N_3Cl$; *Mol. Wt.* 379.919



penta-methyl pararosanilin

$C_{24}H_{28}N_3Cl$; *Mol. Wt.* 393.945



hexa-methyl pararosanilin
(crystal violet)

$C_{25}H_{30}N_3Cl$; *Mol. Wt.* 407.971

(*Basic dyes; absorption maxima: 583–587 in 90% alcohol*)

Solubility at 26°C: in water 2.93%; in alcohol 15.21%

In the case of these compounds, as in the case of other series of homologs differing in extent of methylation, the shade is deepened by the introduction of each methyl group. Hence the various mixtures known to the trade as methyl violet vary from reddish to bluish violets according to the relative amounts of the more and the less completely methylated compounds present in the mixture. This is the significance of the various shade designations listed above, R's indicating the reddish shades, and B's the bluish shades. Of these various shades the bluer ones seem to be best for biological

purposes, methyl violet 2B having been found satisfactory for practically all purposes for which methyl or gentian violet is ordinarily called for. This indicates that the biologist requires the higher homologs in this group. Now the most completely methylated methyl violet is the hexa-methyl compound, which is easily obtained pure and is known to the trade as crystal violet. This dye, in fact, is coming to be the one member of this group in most common use in biology.

Spectrophotometric curves of typical samples of crystal violet and methyl violet 2B are given in Fig. 21. Their great similarity

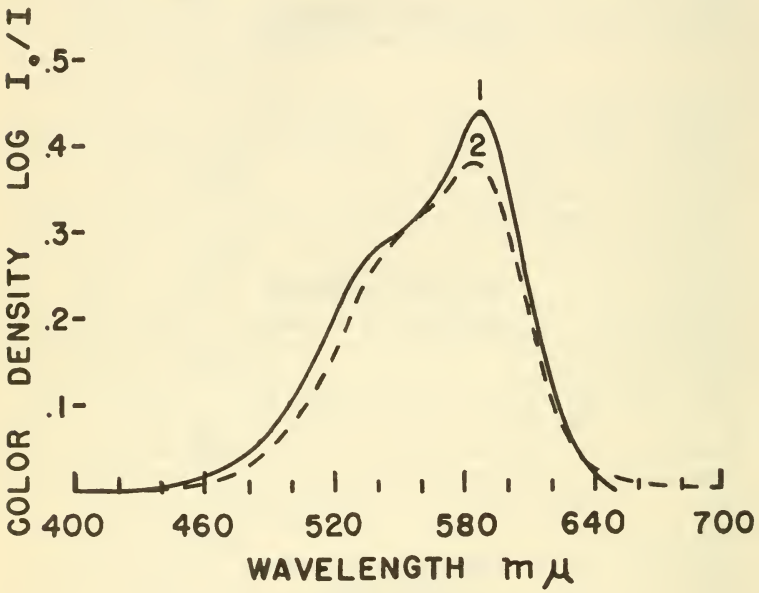


Fig. 21. Spectral curves of two violet rosanilins:
1. Crystal violet. 2. Methyl violet 2B.

is apparent, but the two products can be distinguished by accurate measurement of the absorption maximum.

Gentian violet. A poorly defined mixture of violet rosanilins is well known to biologists under the name gentian violet. The name is not used at present in the dye or textile industries. It apparently applied originally to a certain mixture containing about half dextrin and half dye, the dye being a methyl violet, that is a mixture of crystal violet with lower homologs of the same series. The statement was formerly common in biological literature that gentian violet is a mixture of crystal and methyl violet; but the looseness of the statement is evident when it is realized that crystal

violet is a component of all the deeper shades of methyl violet. It is possible that before 1914 gentian violet did represent a fairly constant mixture, but there seems to be some doubt even on this point. It is certain that immediately following the first World War each company used its own judgment as to what to furnish when gentian violet was ordered. As a result there were placed on the market under this name various methyl violets, with or without dextrin, and also crystal violet; of course the purchaser had no knowledge as to what he was obtaining in any given instance.

Under the circumstances the Commission faced a difficult problem in trying to standardize gentian violet. The question was whether to recognize the name at all, or to approve some particular dye or mixture of dyes of this group as gentian violet. The former course was almost impossible because of the wide demand among biologists for a stain labeled gentian violet; it was plain that the stain companies would meet this demand in some way. The second course (unless considerable latitude were recognized) would be entirely arbitrary, inasmuch as no information was available to show which members of this group of dyes were especially needed in histology or bacteriology. Accordingly in the first edition of this book gentian violet was defined as either penta-methyl or hexa-methyl pararosanilin, or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B.

This definition was quite broad and did not exclude anything sold at that time as gentian violet. As a result there are still various different products sold under this name. The nature of the imported gentian violet has not been recently investigated; but it is known that there are at least three types of gentian violet on the market, prepared by American concerns. They are as follows: 1) crystal violet, as sold under the labels of the National Aniline and Chemical Co. and of the Hartman-Leddon Co., each of these products being plainly marked "crystal violet" in small letters following the name "gentian violet;" 2) one of the higher methyl violets prepared by Coleman and Bell and labeled "gentian violet improved;" 3) a product manufactured by the National Aniline and Chemical Co. and labeled "gentian violet, Churchman," which is claimed to be a mixture in equal parts of crystal violet and methyl violet 2B.

The situation is not yet regarded as entirely satisfactory. Users should specify crystal violet, for bacteriological work and for histological purposes where a deep blue-violet is required; but should order methyl violet 2B in histological procedures where a reddish shade is called for. With this knowledge at hand, gentian violet, as such, is not necessary.

N30 CRYSTAL VIOLET C. I. NO. 681

Synonyms: *Violet C, G,* or *7B. Hexamethyl violet. Methyl violet 10B. Gentian violet.*

$C_{25}H_{30}N_3Cl$; *Mol. Wt.* 407.971

(*A basic dye: absorption maximum 589-593*)

Solubility at 26°C: in water 1.68%; in alcohol 13.87%

This dye is a hexa-methyl-pararosanilin, whose formula is given above as one of the components of methyl violet. The Commission has made as careful an investigation of this dye as of any other and recommends it highly.

Methyl or gentian violet is of chief value to the biologist as a nuclear or chromatin stain, having many histological and cytological applications, the one for which it is most commonly used at present being the Flemming (1891) triple stain in which it is employed with orange G and safranin—a technic which gives a very high degree of cytological differentiation. It is also used for staining amyloid in frozen sections of fresh and fixed tissue, and for staining the platelets in blood while it is much used by the Weigert (1887) technic for staining fibrin and neuroglia. The bacteriologist also finds it a useful stain and probably purchases more at the present time than all other biologists together; the chief bacteriological use is in the Gram technic for distinguishing between different kinds of bacteria. A further more recent use is in bacteriological media for inhibiting the growth of Gram-positive organisms, due to its selective bacteriostatic action. An important example is Petroff's (1915) gentian violet egg medium for isolation and cultivation of the tubercle organism. For most of these purposes, crystal violet is in general use. As a result of its bacteriostatic properties it has come into use as a medicinal agent for the control of staphylococcic infection. It has not yet been definitely established, however, whether crystal violet alone or some mixture thereof with the lower homologs should be used for this purpose.

The Flemming and Gram stains have seemed the most delicate procedures for which it is used; so they have been given the most careful study. In the case of the Gram stain it was discovered that there are a score or more different procedures all referred to by the name "Gram" stain, and a study was made of all the methods that were found (see Hucker and Conn, 1923). The result of the investigation was to conclude without reservation that crystal violet may be substituted for gentian violet in both the Gram and Flemming technics, and probably for gentian or methyl violet in any of the bacteriological or histological methods for which either stain is designated. The advantage of crystal violet is obvious; for it is a definite chemical compound, while methyl and gentian violet are both variable mixtures.

It is of interest to note that in the literature of microscopic tech-

nic crystal violet has been specified instead of gentian violet for some special procedures. Worth noting is Benda's crystal-violet-alizarin method for staining chondriosomes, and its modifications by Meves and Duesberg (1908); and also its use in combination with erythrosin by Jackson (1926) for staining lightly lignified walls, in which technic it proves more uniform than gentian violet.

A loose compound of crystal violet and bismuth, formed by treating an aqueous solution of the dye with an aqueous solution of bismuth ammonium citrate, has been prepared by Wilkinson and Barksdale (1928) and called bismuth violet. It is claimed to have much greater bacteriostatic action on Gram-positive bacteria than crystal violet itself.

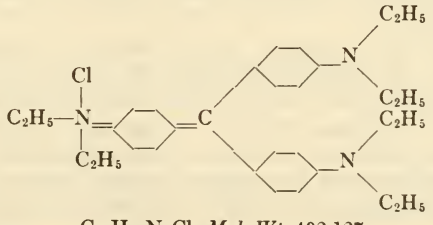
PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Crystal violet stain for amyloid	IA_4-9
Jackson's crystal violet stain for woody tissue	IIA-9
Newton's crystal violet iodine technic for plant cytology	IIB-5
Randolph's crystal violet iodine technic for plant cytology	IIB-5
Permanent smear preparations of sporocytes	IIB-6
Flemming triple stain in plant cytology	IIB-10
Same, with iodine	IIB-11
General staining of pure cultures of bacteria	$IIIA_2$-5
Gram stain: Hucker modification	$IIIA_2$-6
Burke and Kopeloff-Beerman modifications	$IIIA_2$-7
Ljubinski stain for diphtheria organism	$IIIA_2$-12
Anthony's method for bacterial capsules	$IIIA_2$-17
Hiss' method for bacterial capsules	$IIIA_2$-17
Tyler's method for bacterial capsules	$IIIA_2$-18
Gram-Weigert method for bacteria in tissues	$IIIB_3$-8
Lillie modification of Gram stain for tissues	$IIIB_3$-8
Weigert's stain for fibrin and bacteria in tissues	$IIIB_3$-9
MacCallum's stain for influenza bacilli in tissues	$IIIB_3$-14

N35 ETHYL VIOLET C. I. NO. 682

Synonym: *Ethyl purple 6B.*

Ethyl violet is hexa-ethyl pararosanilin having the following formula:



$C_{31}H_{42}N_3Cl$; *Mol. Wt.* 492.127

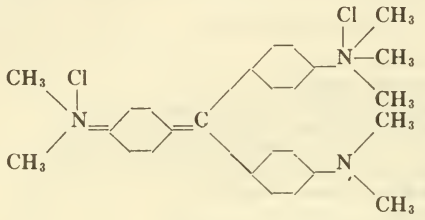
(*A basic dye; absorption maximum about 596*)

It has been called for by Bowie (1924) in a neutral stain combination with Biebrich scarlet to stain the islets of Langerhans. Kernohan (1931) and Proescher (1934) have each employed it in staining nervous tissue; while Ono (1934) uses it for staining spirochaetes in blood.

N40 METHYL GREEN C. I. NO. 684

Synonyms: *Double green SF. Light green.*

Methyl green is crystal violet into which a seventh methyl group has been introduced by the action of methyl chloride or methyl iodide upon it, forming the compound:*



$C_{26}H_{33}N_3Cl_2$; *Mol. Wt.* 458.462

(*A basic dye; absorption maximum 630–634,* [*420*])

As the seventh methyl group is very loosely attached, there is always some methyl or crystal violet present, either because it is not all converted into the higher homolog or because it has broken down again. It has been stated that to obtain free methyl green the commercial dye should be shaken in a separatory funnel with amyl alcohol or chloroform, which dissolves the methyl violet. As a matter of fact, however, pure methyl green may not always be desired by the biologist, as the dye owes part of the metachromatic properties for which it is prized to the presence of small amounts of the violet compound.

Methyl green is at present one of the most valuable nuclear stains known to the histologist, and is widely used as a chromatin stain by the cytologist. On the other hand it has been used by Galeotti as a cytoplasm stain following acid fuchsin and picric acid; (see Krause, 1926–7, p. 1417). In the Ehrlich-Biondi technic it is used to stain nuclei in contrast to acid fuchsin; (Id. p. 457); while Bensley employs it to stain chromatin in contrast to acid

*This ordinarily occurs in trade as a zinc chloride double salt.

fuchsin which stains the mitochondria; (see Lee, 1937, p. 166). It is an ingredient of the Ehrlich triacid mixture (with orange G and acid fuchsin) for staining blood smears. Botanists find it a valuable stain, combined with acid fuchsin, for lignified xylem. One of its most valuable uses today is in the Pappenheim (1899) stain, in which it is combined with pyronin and used for staining the gonococcus and mast cells as well as by Unna in studying chromolysis. It is also a useful chromatin stain for protozoa, and is employed in weak acetic acid solution for staining fresh material beneath the cover glass.

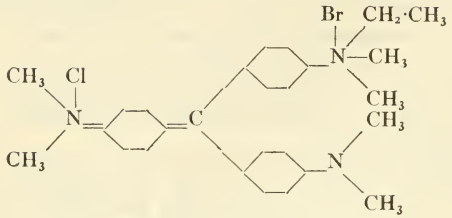
When the foreign supply of dyes was first shut off, this stain proved one of the most difficult to obtain in satisfactory quality, largely due to the looseness with which the seventh methyl group is attached and the resulting instability of the compound. At first certain green dyes of an entirely different nature were furnished, but as soon as an investigation of the dye was begun manufacturers proved perfectly able to produce methyl green; the difficulty came in obtaining the right degree of purity. Samples were finally furnished so pure that they lacked completely the necessary metachromatic staining quality; and it proved necessary to add a certain small percentage of the violet dye to obtain the proper results. This problem seems to have been solved at present and satisfactory methyl green is available. The chief problem now is to standardize it. With other stains this can ordinarily be done on the batch basis, approving some batch large enough to meet the demand for a period of years. With methyl green this cannot safely be done, on account of its instability. Hence large batches are impractical; and the stain ought to be sold with the caution that the dye does not keep indefinitely without change.

This instability complicates standardization by spectro tests. Fig. 19, p. 145, shows a spectrophotometric curve of this dye in comparison with those of light green SFY and fast green FCF. The three are much alike with two maxima at about 430 and 630. All the green dyes show disagreement between dye content determinations by means of $TiCl_3$ titration and comparisons of the density at the peak of the chief maximum. Methyl green has an additional source of inconsistency in its assay: the ratio between two points of the curve equal distances each side of the maximum fluctuates, due apparently to the fact that solutions change on standing.

There seems, since 1930, to have been a change in the nature of the methyl green samples supplied as biological stains. One of the companies admits that its methyl green is not C. I. No. 684, but the following:

N42 ETHYL GREEN C. I. NO. 685

Synonym: *Methyl green.**



$C_{27}H_{35}N_3Cl\,Br$; *Mol. Wt.* 516.947

(*A basic dye*)

This dye differs from the preceding only in that it is prepared from crystal violet by the action of an ethyl instead of a methyl halide. It is almost, if not entirely, indistinguishable from C. I. No. 684 by spectrophotometric tests, and apparently acts very similarly for biological purposes. It is entirely possible that it is even better than the other for use in staining; certainly the present methyl greens are more satisfactory than those on the market between 1920 and 1930.

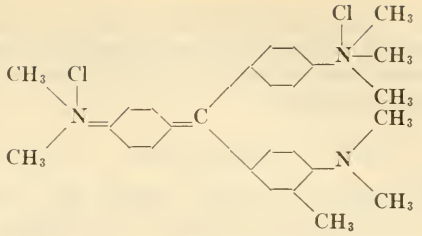
PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH EITHER METHYL GREEN OR ETHYL GREEN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Bismarck brown with methyl green in animal histology	IA_1-5
Methyl green in glycerin jelly for pollen grains	IIA-13
Albert's stain for the diphtheria organism	$IIIA_2$-11
Pappenheim-Saathof stain for gonorrheal pus	$IIIA_2$-19
Saathof stain for bacteria in tissue	$IIIB_3$-5

N45 IODINE GREEN C. I. NO. 686

This dye is closely related to methyl green, the generally accepted formula being:

*This ordinarily occurs in trade as a zinc chloride double salt.



$C_{27}H_{35}N_3Cl_2$; *Mol. Wt.* 472.488

(*A basic dye*)

Iodine green is a nuclear or chromatin stain which has selective properties that make it of value in certain special procedures. It was used by Ciaccio (1906) for staining nervous tissue, in combination with acid fuchsin and picric acid; by Lefas (see Krause, 1926–7, p. 1122) as a blood stain in combination with acid fuchsin; and by others as a stain for mucin and amyloid which it colors red instead of green. It was employed by Zimmermann (1893) with basic fuchsin for staining chromatin in plant tissue; while together with acid fuchsin it has been proposed by other botanists for staining lignified xylem. Torrey and Buckell (1922) have recommended a differential medium for the isolation of the gonococcus which includes this dye.

ACID VIOLET

There are various dyes on the market known as acid violet with some shade designation. These dyes vary extremely in composition and they are listed under various Colour Index numbers. They are in general sulfonated violet dyes of the rosanilin group, some of them simple methylated pararosanilins, others benzylated compounds.

Some acid violet—its exact identity uncertain—has been employed by Bailey (1921) in cytological studies on the human pituitary gland; also by Maurer and Lewis (1922) for staining similar tissue from the pig; by Ono (1934) for staining spirochaetes in blood, and by Weiss for staining both spirochaetes (1929) and bacterial flagella (1928). It is very unfortunate that any biologist should have been furnished a stain labeled merely acid violet; the term is too indefinite for identification.

BENZYL VIOLET

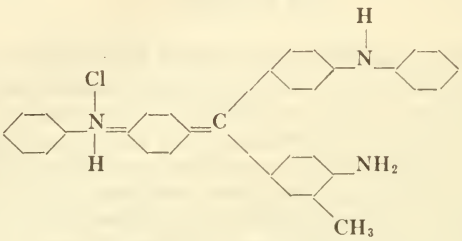
There is a group of dyes known as benzyl violets, which are pararosanilins with benzyl substitution in one or more of the

amino groups. Some of them are acid and some basic dyes. There is occasional reference in the biological literature to the use of a benzyl violet, but without any indication as to which of the dyes in question is intended.

N50 SPIRIT BLUE C. I. NO. 689

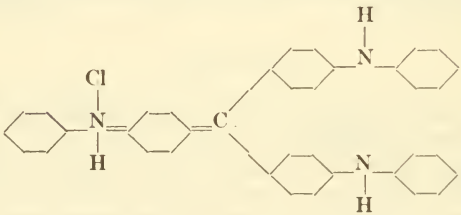
Synonyms: *Gentiana blue 6B. Anilin blue, alcohol soluble. Light blue. Lyon blue. Paris blue.*

This is a mixture of di-phenyl rosanilin chloride:



$C_{32}H_{28}N_3Cl$; *Mol. Wt.* 490.025

and tri-phenyl pararosanilin chloride:



$C_{37}H_{30}N_3Cl$; *Mol. Wt.* 552.091

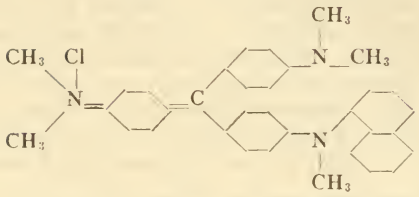
(*Basic dyes absorption maximum of spirit blue 2R about 581 in alcohol.*)

Solubility at 26°C: in water nil; in alcohol 1.10%

Lynch (1930) mentioned Lyons blue (undoubtedly this dye) as a satisfactory counterstain to carmine in bulk staining of protozoa and other small animals and embryos. It has, moreover, been reported by others as a good contrast stain for carmine, bringing out nerve fibers well in embryonic tissue; for this purpose it is used in strong alcoholic solution. Quite recently, Knaysi (1942) has mentioned this dye as one whose color base can be used in determining hydrolysis of fats.

N55 VICTORIA BLUE 4R C. I. NO. 690

Synonym: *Fat blue 4R.*



$C_{34}H_{34}N_3Cl$; *Mol. Wt.* 520.093

(*A basic dye; absorption maxima 593.5* [*538.5*])

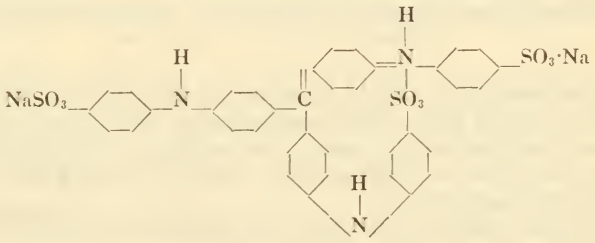
Solubility at 26°C: in water 3.23%; in alcohol 20.49%

Herzberg (1934) suggested this dye for staining minute granules in the case of certain virus diseases, claiming these granules to be the filterable virus itself. Others (e.g. Gutstein, 1937) have followed the same technic, staining the "elementary bodies", but without committing themselves to Herzberg's theory. Isada (1938) mentioned this dye as one of several that may be used in staining bacterial flagella. Lipp (1940) has proposed it, either alone, or mixed with methyl green and pyronin, for staining spirochaetes, particularly *Treponema pallidum.*

The various Victoria blues are often confused; see also Victoria blue R and B, a few pages below.

N60 METHYL BLUE C. I. NO. 706

Synonyms: *Cotton blue. Helvetia blue.*



$C_{37}H_{27}N_3O_9S_3Na_2$; *Mol. Wt.* 799.784

(*An acid dye; absorption maximum about 607*)

On account of the sulfonic groups, this dye is strongly acidic and makes a good counterstain. It may, for instance, be substi-

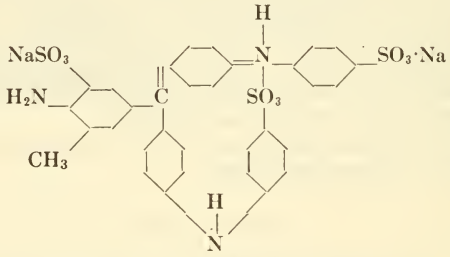
tuted for anilin blue W.S., as in the Mallory connective tissue stain. Such uses of this dye are too numerous to list, but one might mention that of Dubreuil (see Krause, 1926-7, p. 1382) who employed it, combined with picric acid, in contrast to a red nuclear stain such as carmine or safranin. One of its oldest and best known uses is that of Mann (1894) who mixed it with eosin and showed the value of the mixture in staining nerve cells. Recent workers suggest it for other purposes: Clauser and Strani (1930) for staining unfixed tissue; Cumley (1935) for the negative staining of bacteria; Monné (1935) in the vital staining of protozoa.

For use in **buffered Mann stain**, see Staining Procedures p. IA_4-19; in technic **with Biebrich scarlet** see p. $1B_3$-12.

N65 ANILIN BLUE, W. S. (i.e., water soluble) C. I. NO. 707

Synonyms: *China blue. Soluble blue 3M or 2R. Marine blue V. Cotton blue. Water blue.*

This is a mixture of the tri-sulfonates* of tri-phenyl para-rosanilin (C. I. 706) and of di-phenyl rosanilin. The latter is:



$C_{32}H_{25}N_3O_9S_3Na_2$; *Mol. Wt.* 737.718

(*An acid dye; absorption maximum of samples submitted for certification 595–610 in alcohol*)

Anilin blue W. S. should be regarded as a group of dyes rather than as a simple dye. The composition of the various commercial products sold under this name is uncertain, and no method seems to be known for controlling the process of manufacture so as to yield a constant product.

The spectrophotometric curve of a sample typical of those sold as biological stains is given in Fig. 22.

Certain dyes of this group (apparently only the rosanilin derivatives, because they are most likely to be free from undesirable colored impurities), have indicator properties, decolorizing almost completely on addition of alkali, and slowly becoming blue again

*The location of the sulfonic groups is uncertain.

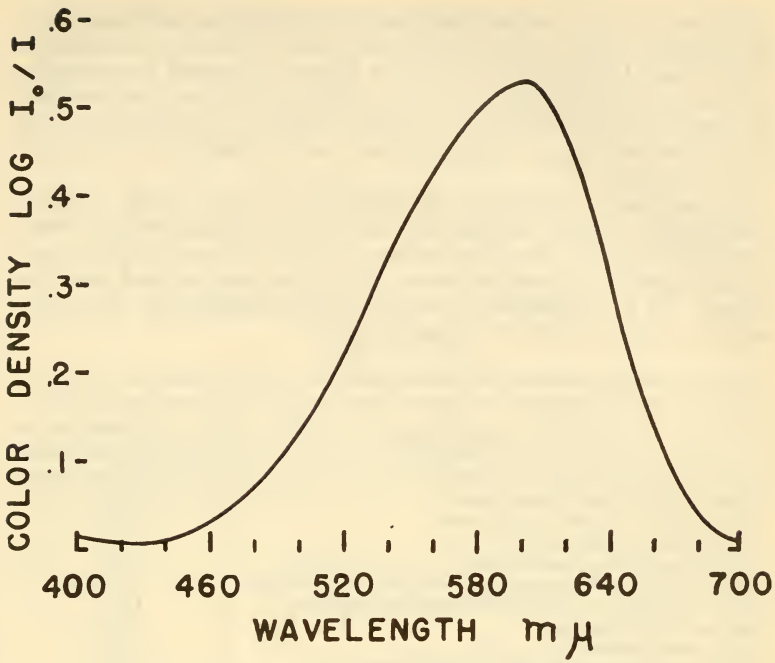


FIG. 22. Spectral curve of anilin blue W.S.

if the reaction becomes acid. It is not a specially valuable indicator, however, partly because of the slowness with which it responds to change in reaction, and partly because it is quite subject to protein and other errors. In spite of these drawbacks it has sometimes been used in bacteriological media, when accuracy is not important and the slow response to acid production is of no significance.

Hendrickson, Baldwin, and Riker (1934) have employed it in bacteriological media for a different purpose, the isolation of the crown gall organism; in their medium it holds fungi in check while the desired organism absorbs the blue color and is thus easy to recognize. For some reason not yet explained, those samples (apparently pararosanilin derivatives) which do not decolorize properly in alkaline solution, fail to work in this medium, although the dye does not serve in this instance as an acid-base indicator.

By far the best known use of anilin blue, W.S., is as a counterstain in histology. Among its histological applications are: by Stroebe and Huber (see Krause, 1926-7, p. 63) as a cytoplasm stain preceding safranin; by Galli for axis cylinders; frequently by botanists as a contrast for safranin in vascular plant tissue, or for

erythrosin or phloxine in algae; and very widely by pathologists in the Mallory (1900) connective tissue stain, in which it is combined with orange G and acid fuchsin; by Unna in contrast to orcein for staining epithelial sections (see Lee, 1937, p. 438) and in studying the process of chromolysis; by Koneff (1936) in combination with iron hematoxylin as a polychrome staining method for routine laboratory use.

On account of the lack of constancy in this group, anyone publishing a technic calling for one of these dyes should be very careful to give all the information obtainable from the label and should specify the source from which the sample used was obtained. If a Commission certified stain is employed, its certification number should of course be given.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Mallory's anilin blue collagen stain	IB_3-3
Heidenhain's "azan" modification	IB_3-4
Crossman's modification	IB_3-5
Acid alizarin blue modification	IB_3-6
Biebrich scarlet and picro-anilin-blue in animal histology	IB_3-12
Lillie modification of Gallego elastic tissue stain	IB_3-15
Masson's trichrome stain	IB_3-18
Lillie modification	IB_3-19
Safranin and picro-anilin-blue in plant histology	IIA-11
Safranin and anilin blue in plant cytology	IIB-12

N70 ISAMINE BLUE C. I. NO. 710 (?)

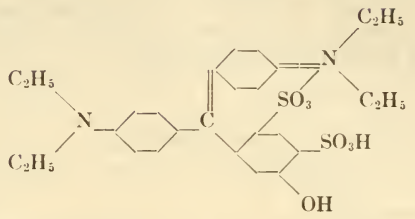
Occasional reference is made, especially in the European literature, to a dye called isamine blue. Its exact chemical nature is not at present known, although the Colour Index lists isamine blue 6B or 8B as a synonym of No. 710 (alkali blue XG) and isamine blue B and R as similar to this dye. The dye is not yet manufactured in America but was available from German sources before the second World War. It is known to be an acid dye, a sulfonated naphthyl-rosanilin or naphthyl-pararosanilin.

Isamine blue is employed to some extent in Europe as a vital dye by intravenous injection, followed by lead salts or radiation. Exact references to such uses, however, are not now available. Dean (1937) employs it for determining the proportion of antigen to antibody in immune sera.

N75 PATENT BLUE V C. I. NO. 712

Synonyms: *Alphazurine 2G. Patent blue VF. Pontacyl brilliant blue V.*

Usually the calcium salt of the following:



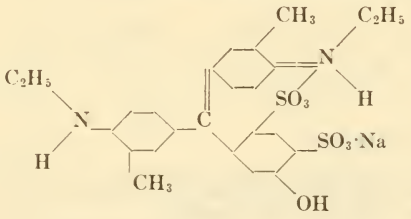
$(C_{27}H_{31}N_2O_7S_2)_2$ Ca; *Mol. Wt.* 1159.388

(*An acid dye*)

This dye has rarely been mentioned in biological literature; but is included by Ono (1934) among those employed for staining spirochaetes; it is mentioned by McMaster and Parsons (1938) as a vital stain, and by Yoe and Boyd (1939) as an acid-base and oxidation-reduction indicator.

N80 XYLENE CYANOL FF C. I. NO. 715

Synonym: *Cyanol FF*



$C_{25}H_{27}N_2O_7S_2Na$; *Mol. Wt.* 554.599

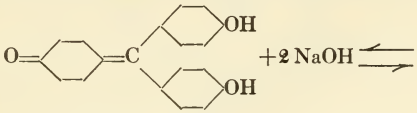
(*An acid dye*)

Chambers (1935) has included this among several dyes which he studied in connection with the uptake of dyes by cells in tissue culture. A cyanol, probably this one, has been used by Fautrez (1936) in histochemistry, as an intracellular indicator; and by Ivanov and Braun (1938) in the study of permeability of tissue membranes.

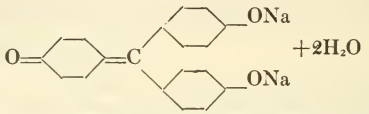
c. Hydroxy Tri-phenyl Methanes (Rosolic Acids)

The rosolic acid dyes, as stated above, are tri-phenyl methane derivatives in which the amino groups of the rosanilins are replaced with hydroxyl groups, thus giving them acidic instead of

basic character. The compounds of this group are not very important as dyes and are scarcely used as stains. The greatest interest of the biologist in them is due to their use as indicators. In acid solution rosolic acid is yellow but it is converted by alkali to the disodium salt which is red, thus:

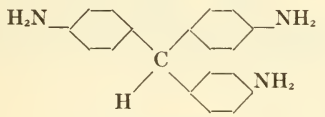


pararosolic acid

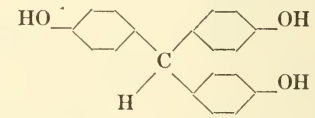


disodium pararosolate

There is considerable confusion in the nomenclature of these dyes, as the names employed may be used in a strict chemical sense or in a looser sense in practice. Chemically there are two rosolic acids, which are related just as are rosanilin and pararosanilin. Pararosolic acid differs from pararosanilin, only in having hydroxyl groups in place of the amino groups:

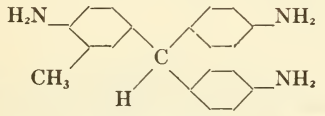


leuco-pararosanilin

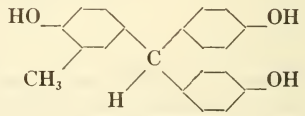


leuco-pararosolic acid

Rosolic acid, on the other hand, is a mono-methyl derivative, and bears the same relation to rosanilin:



leuco-rosanilin



leuco-rosolic acid

Now the dye to which the name rosolic acid or aurin is generally given in practice is a mixture consisting of both rosolic acid and pararosolic acid together with other closely related compounds. This dye is:

N90 AURIN OR ROSOLIC ACID C. I. NO. 724

The above names apply to the free acid. *Yellow corallin* is the sodium salt.

A mixture of rosolic acid and pararosolic acid, with oxidized and methylated derivatives of the latter. This product is of considerable use as an indicator.

This dye was employed by Pappenheim (1898) in a decolorizing solution following carbol fuchsin. The method was intended to safeguard against confusion of the smegma and other acid-fast bacteria with the true tubercle bacillus. After the usual carbol fucnsin stain, the decolorizing was done with 1% rosolic acid in absolute alcohol, saturated with methylene blue and containing 20% glycerol.

No other dyes of this group have biological use. Two others perhaps deserve mention:

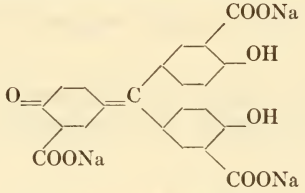
N93 RED CORALLIN C. I. NO. 726

Synonym: *Aurin R.*

Probably a pararosolic acid salt of pararosanilin, forming an intermediate stage in the conversion of pararosolic acid into pararosanilin.

N95 CHROME VIOLET CG C. I. NO. 727

A carboxyl derivative of pararosolic acid:



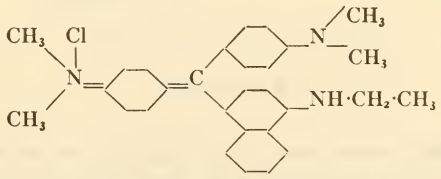
$C_{22}H_{11}O_9Na_3$; *Mol. Wt.* 488.299

(*An acid dye*)

3. DIPHENYL-NAPHTHYL METHANE DERIVATIVES

P5 VICTORIA BLUE R C. I. NO. 728

Synonyms: *New Victoria blue B or R. Corn blue B.*



$C_{29}H_{32}N_3Cl$; *Mol. Wt.* 458.027

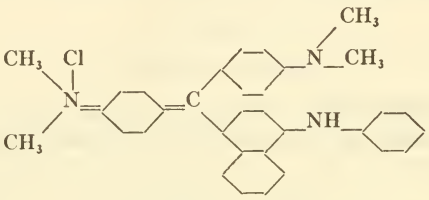
(*A basic dye; absorption maxima 614.7 [558]*)

Solubility at 26°C: in water 0.54%; in alcohol 3.98%

Some Victoria blue, possibly this or the following or possibly C. I. No. 690, has been employed by Gutstein (1932) as a vital stain for yeast, by Hausdorff (1927) for staining spermatazoa in the testicle, and by Ono (1934) for staining spirochaetes in blood. The various Victoria blues are frequently confused in the literature.

P10 VICTORIA BLUE B C. I. NO. 729

Synonyms: *Fat blue B. Corn blue BN.*

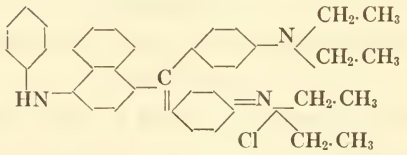


$C_{33}H_{32}N_3Cl$; *Mol. Wt.* 506.067

(*A basic dye; absorption maxima 619.2* [*567*])

This dye has been employed by Proescher (1934) for staining neuroglia, and its eosinate by Geschickter (1930a) for fresh frozen tissue.

P11 NIGHT BLUE C. I. NO. 731



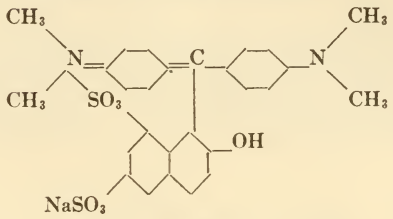
$C_{37}H_{40}N_3Cl$; *Mol. Wt.* 562.171

(*A basic dye*)

Hallberg (1946) recommended this dye in place of carbol fuchsin as a stain for acid fast bacteria, with phenolic pyronin as a counterstain. The procedure has been followed to some extent in Europe, but is not much used in America. The dye has recently become hard to obtain commercially.

P15 WOOL GREEN S C. I. NO. 737

Synonyms: *Wool green BS, BSNA or C. Lissamine green B, BS. Pontacyl green S. Calcoid green S extra. Acid green S. Cyanol green B. Fast light green.*



$C_{27}H_{25}N_2O_7S_2Na$; *Mol. Wt.* 576.603

(*An acid dye; absorption maximum 634*)

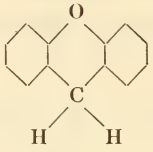
This dye has been mentioned by Lillie (1940, 1945) as a satisfactory substitute for fast green FCF in his modification of the Masson trichrome stain.

For use in **Lillie modification of Masson trichrome stain,** see Staining Procedures, p. IB3-19.

CHAPTER VIII

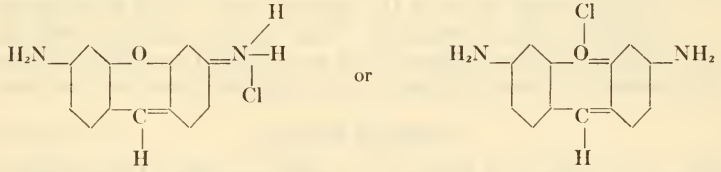
THE XANTHENE DYES

THE group of compounds known as xanthene dyes comprises a number of basic and acid dyes and quite a series of indicators. In fact, the most valuable indicators known to the chemist fall in this group. They are derivatives of the compound xanthene:

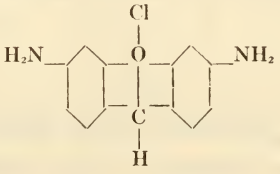


1. THE PYRONINS

The pyronins are methylated di-amino derivatives of xanthene. They are closely related to the diphenyl methanes and are sometimes classed with them, as they have a carbon atom attached to two benzene rings, and show the same tendency toward quinone structure. Their formula, on the other hand, is like that of the oxazins except that the nitrogen of the central ring is replaced by a methenyl (CH) radical. Like the oxazins, the atomic grouping may be assumed to be in either the paraquinoid or the ortho-quinoid form, thus:



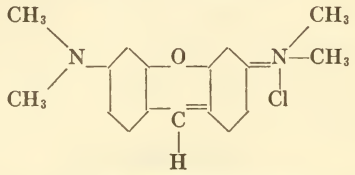
Another arrangement of the atoms is possible in which no quinoid ring exists, namely:



This latter form might also be assumed for the oxazins and thiazins as well, and this type of formula is frequently used for the azins; but the xanthene dyes are more often represented in this form. If this formula is adopted the quinoid ring cannot be accepted as their chromophore. For this reason one of the quinoid formulae seems preferable; and for the sake of uniformity the paraquinoid form will be given in the following pages. It must be remembered, however, that the other formulae are equally admissible; and it is possible that the compounds occur in two or even all three of the different forms.

Q5 PYRONIN Y C. I. NO. 739

Synonym: *Pyronin G.*



$C_{17}H_{19}N_2OCl$; *Mol. Wt.* 302.795

(*A basic dye; absorption maximum about 552*)

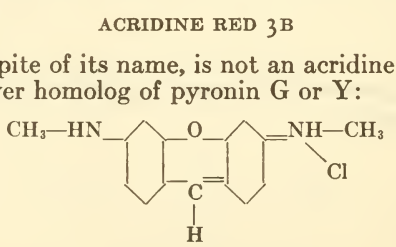
Solubility at 26°C: in water 8.96%; in alcohol 0.60%

This dye, finding occasional application as a biological stain in pre-war days, was not manufactured in America until 1931. For most purposes pyronin B may be substituted for it; but Scudder (1931) finds it necessary in her combined Gram-Pappenheim stain.

A typical spectral curve of this dye is given in Fig. 23, in which it is compared with pyronin B. This graph, together with the others in this chapter, is from Stotz *et al.* (1950) in which the spectrophotometric characteristics of the xanthene dyes are discussed.

Q8 ACRIDINE RED 3B C. I. NO. 740

This dye, in spite of its name, is not an acridine derivative, but a pyronin, a lower homolog of pyronin G or Y:



$C_{15}H_{15}N_2OCl$; *Mol. Wt.* 274.743

(*A basic dye; absorption maxima: [508] 547.5*)

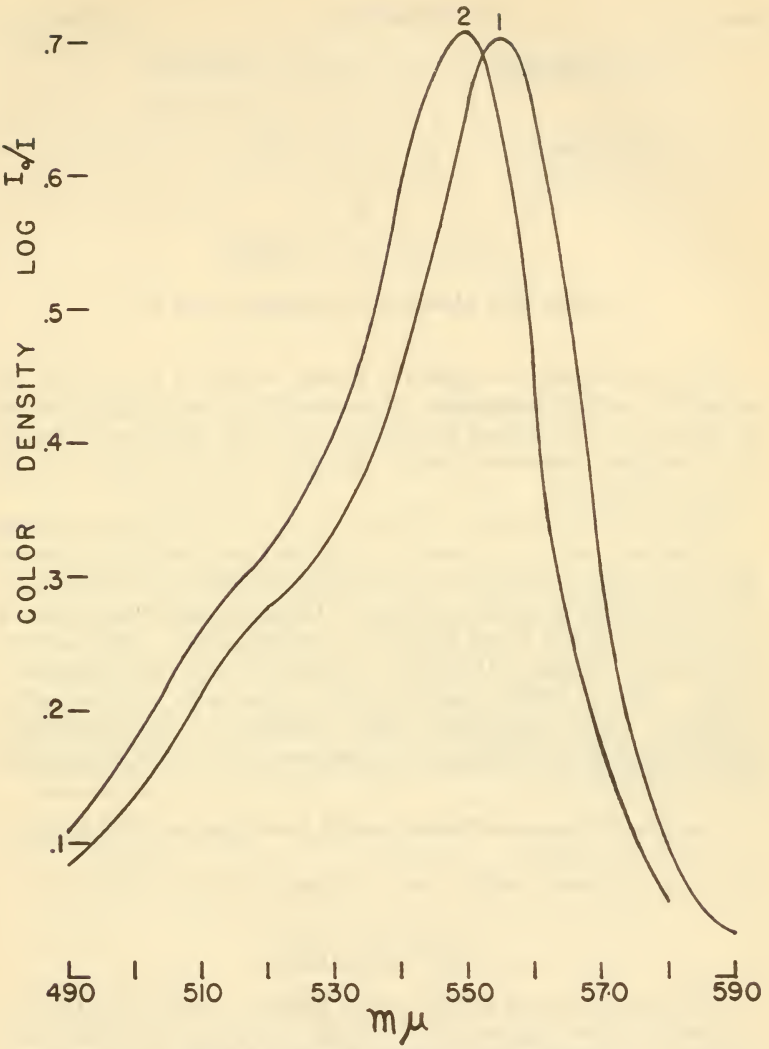
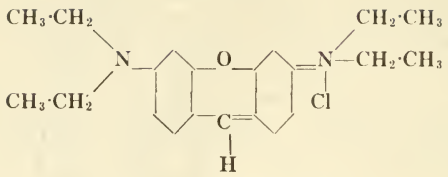


FIG. 23. Spectral curves of two pyronins:
1. Pyronin B. 2. Pyronin Y.

It is rarely called for in biological work, but has been used by Gömöri (1943), mixed with methyl green, in a procedure for staining tissue to demonstrate deposits of calcium salts and sites of phosphatase activity.

q10 PYRONIN B C. I. NO. 741



$C_{21}H_{27}N_2OCl$; *Mol. Wt.* 358.899

(*A basic dye; absorption maximum about 555*)

This dye differs from pyronin Y only in that it is an ethyl instead of a methyl derivative. As a result it is very slightly deeper in shade but has almost identically the same staining behavior. The two dyes have very similar spectral curves, as shown by the graphs in Fig. 23.

The pyronins find their principal use in the Pappenheim (1899) combination, where a pyronin is employed with methyl green for staining basophile elements, especially the mast cells, and for staining the gonococcus in smears of pus. It is also used sometimes as a counterstain in the Gram technic for bacteria (see p. 160); and by Ehrlich and Lazarus (1898) as a component of certain "neutral" stains. Monné (1938) has employed a pyronin for staining mitochondria; while Huddleson (1931) makes use of its bacteriostatic properties in distinguishing between species of the bacterial genus, *Brucella*.

For technic of **Pappenheim-Saathof stain for gonorrhoeal pus**, see Staining Procedures,* p. IIIA$_2$-19.

For technic of **Saathof stain for bacteria in tissues**, see Id. p. IIIB$_3$-5.

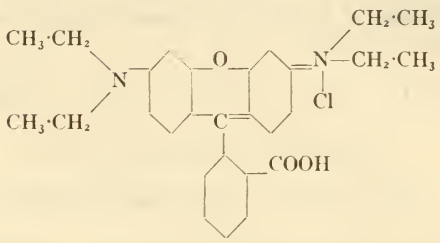
2. THE RHODAMINES

The rhodamines are similar to the pyronins except that there is a third benzene ring attached to the central carbon atom and attached to this ring is a carboxyl group in the ortho position. This latter group, although of acid tendency, does not counteract the basic action of the amino groups, so the dyes are basic in character.

*Conn and Darrow (1943-4).

Q20 RHODAMINE B C. I. NO. 749

Synonyms: *Rhodamine O. Brilliant pink B.*



$C_{28}H_{31}N_2O_3Cl$; *Mol. Wt.* 479.001

(*A basic dye; absorption maxima about 556.5,* [*517*])

Solubility at 26°C: in water 0.78%; in alcohol 1.47%

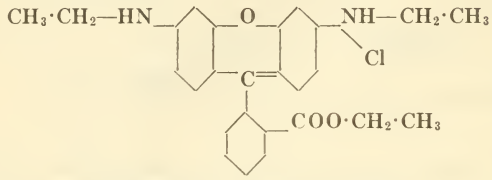
A rhodamine, probably the above dye, has been used by Griesbach with osmic acid to fix and stain blood simultaneously; by Ehrlich as a component of "neutral" stain mixtures; also for histological work in contrast to methylene blue; and by others in contrast to methyl green. (See Krause, 1926-7, p. 2023.) Although these early users were not always careful to specify which rhodamine had been employed in their work, rhodamine B has been definitely specified by later workers, e.g.: by Houcke (1928) as a stain for glandular tissue, when mixed with methylene blue; by Strugger (1938) as a vital stain for *Allium* epidermis, calling attention to its fluorescence under ultraviolet light; by Monné (1939) in studies on the Golgi apparatus; by Levaditi *et al.* (1940), as a fluorochome of value in virus studies; by Metcalf and Patton (1944), as a fluorochrome for use in insect histology; and by Frederick (1941), as a microchemical reagent for the estimation of antimony. It seems to be coming into more common use at present, thanks in good part to its fluorescent properties in ultraviolet light.

As a rhodamine is not infrequently specified without a shade designation, it is sometimes not possible to be sure that the above dye is intended. If not, the following is the dye most probably indicated.

Q25 RHODAMINE 6G C. I. NO. 752

Synonyms: *Rhodamine 6GX and 6GDN extra.*
Calcozine red 6G extra.

By esterification of a compound closely related to rhodamine B, the following is obtained:



$C_{26}H_{27}N_2O_3Cl$; *Mol. Wt.* 450.949

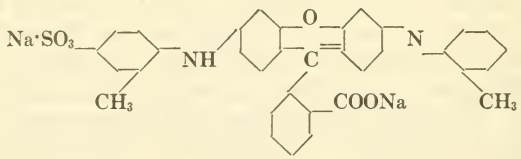
(*A basic dye; absorption maxima* [*492*] *526*)

This dye has not been specified as frequently as rhodamine B, but is mentioned by Strugger and by Monné (cited above), each of whom employed both dyes. Whether this ethyl ester has any advantage over Rhodamine B is not stated.

A somewhat different dye, known as Rhodamine S (C. I. No. 743) has been mentioned in the same connection and may have been used for some of the above-mentioned purposes. It is not a true rhodamine, however, but belongs to a closely related group of compounds, the succineins; for it does not have the three benzene rings, the radical $C_6H_4 \cdot COOH$ being replaced by $C_2H_4 \cdot COOH$.

Q26 VIOLAMINE R C. I. NO. 758

Synonyms: *Acid violet 4R. Fast acid violet 3RL, A2R and R.*



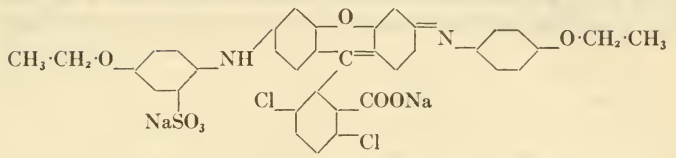
$C_{34}H_{24}O_6N_2SNa_2$; *Mol. Wt.* 634.608

(*An acid dye*)

Lillie (1945 c) has mentioned this dye, among others, as a stain for collagen in tissue sections.

Q30 FAST ACID BLUE R C. I. NO. 760

Almost the same as: *Violamine 3B.*



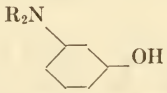
$C_{36}H_{26}N_2O_8SNa_2Cl_2$; *Mol. Wt.* 763.552

(*An acid dye; absorption maximum 534*)

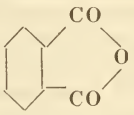
The dye indexes (both Schultz and the Colour Index) list violamine 3B as almost synonymous with this. Violamine 3B, however, is a mixture containing a small amount of some red dye. Some samples of fast acid blue R, made for textile application, are also mixtures, although pure samples have been obtained. It is hoped that pure dye of this type may become regularly available for use as a biological stain.

This dye has been recommended by Romell for use in phenolic solution for staining bacteria in soil. In the case of soils (mucks, forest soils, etc.) containing much brownish or reddish matter, this blue dye gives greater visibility of the microörganisms than dyes of the eosin series.

In practice the rhodamines are prepared not from xanthene but by the condensation of two molecules of dialkyl meta-amino phenols,



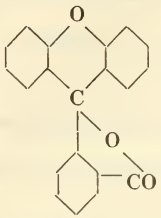
with one of phthalic anhydride:



This shows their close relation to the next group of dyes, namely the fluoran derivatives, which as will be seen are also prepared from phthalic anhydride. In fact these two groups of dyes, acid and basic respectively, are related in exactly the same way as the rosolic acids and the rosanilins, the one group having hydroxyl radicals where the other has amino groups.

3. FLUORAN DERIVATIVES

Fluoran is not a dye, but is a very important compound in dye chemistry. It is a derivative of phthalic anhydride, and contains a xanthene ring (five C atoms and one O atom) as well as a lactone ring (four C atoms and one O atom) besides three benzene rings; thus:



The fluoran dyes are derivatives of this by the introduction of hydroxyl groups into two of the benzene rings at the para position to the central carbon atom and the further introduction of halogen atoms at various positions in all three benzene rings.

It proves convenient here to class these compounds with the xanthene dyes. They may, however, be equally well considered tri-phenyl-methane dyes, as can be seen by a glance at the formula of any of them; in fact they are generally so considered by the chemists. To the biologist they stand in a distinctly different class from the tri-phenyl-methanes; and for that reason are treated here instead of in the preceding chapter. The dyes of this group are also, and equally correctly, spoken of as the fluorescein derivatives, fluorescein being, as will be seen on p. 186, a salt of di-hydroxy-fluoran. Sometimes, moreover, the fluorescein dyes are referred to as the eosins. This term to cover the whole group is hardly justified, as it is better to call eosins only the dyes definitely so named below.

The fluorescein dyes are all similar in their action, but with certain decided differences. In order for the reader to obtain a real understanding of these stains and their behavior, a general discussion of the whole group is necessary.

The dyes in this group most important to the biologist are: eosin Y, C. I. No. 768 (also called eosin yellowish or eosin water soluble); ethyl eosin, C. I. No. 770 (also called eosin alcohol soluble); eosin B, C. I. No. 771 (also called eosin bluish); erythrosin B, C. I. No. 773; phloxine, C. I. No. 774; rose bengal, C. I. No. 779. It is not yet absolutely certain which of these dyes is most suitable for any particular purpose; but some information is available on the subject. The difference in behavior of the different dyes of this group seems to depend upon two factors: difference in color and difference in acidity. The relation between these two factors and chemical structure was discussed by Conn

and Holmes (1926); by reference to which paper it will be seen that in color the dyes above listed increase in depth in the following order: eosin Y, ethyl eosin, eosin B, erythrosin B, phloxine, rose bengal. In the case of each of these, the color is lighter or deeper according to the number of halogen atoms present. Eosin B is the strongest acid in the group, and eosin Y the next strongest; erythrosin is weaker, phloxine weaker than eosin and possibly weaker than erythrosin; while rose bengal is the weakest of all.

To interpret this information into terms of actual use, it is necessary to remember that there are two main types of histological procedures in which eosin is used: first as a general counterstain (usually in alcoholic solution) following a basic dye; secondly, as a cytoplasm stain (always in aqueous solution) preceding a basic dye. In the first of these types of procedure it is important that the dye be one with diffuse staining properties and with a color showing good contrast to the basic dye employed (generally methylene blue or hematoxylin). The more acid and lighter colored dyes in the series (eosin Y, ethyl eosin, and eosin B) seem to possess these properties to the greatest extent and accordingly to be best adapted to such procedures. The particular dye to choose depends undoubtedly on the exact shade desired.

The second type of procedure (i.e. preceding a basic dye) is represented by the Mallory phloxine-methylene-blue stain and the Held erythrosin-methylene-blue technic. In such procedures as these, both dyes are used in aqueous solution; and there is therefore much more opportunity for chemical interaction between the acid dye already in the tissue and the basic dye subsequently added than when the acid dye is used in alcoholic solution. (It is well known that acid and basic dyes in alcoholic solution do not form insoluble reaction products.) In procedures like the Mallory technic, there seems to be a tendency (possibly on account of this interaction) for the acid dye to stain the cytoplasm too weakly when followed by a basic dye, in case the very acid eosin Y is used. Thus Held employs erythrosin, and Mallory who formerly specified eosin discovered later that his original eosin was not a true eosin Y, phloxine giving better results in his technic than one of the true eosins. Now phloxine and erythrosin are not only deeper in color than eosin Y, but are also less strongly acidic; and it is possible that their chemical nature rather than their color may be the decisive factor in determining their superiority for such procedures as this. Rose bengal is even deeper in color, and is still less strongly acidic; in fact, it seems to be too purple to contrast well with methylene blue and of such a weakly acid character that it tends to remain in the nuclei when used by the technic in question. It might prove valuable preceding some basic dye; but it has been found to give poor results preceding methylene blue in the Mallory technic.

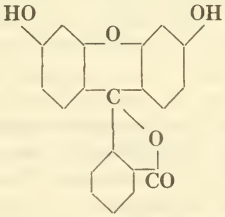
In the procedure for staining bacteria in soil, as developed by Conn (1918) and by Winogradsky (1924), the matter is still further complicated by the fact that the dye must be of such a color and such a strong acid as to stain the bacteria, but not the dead organic matter present.

Another factor of much importance in the staining action of these dyes is the amount of mineral salt present. It has, in fact, been shown by Conn and Holmes (1928) that the intensity with which a dye of this group can stain bacteria may be greatly increased by adding a minute amount (0.001 to 0.1%) of some mineral salt such as $CaCl_2$. This matter is discussed in Chapter II.

There has been in the past much mislabeling of the dyes of this group. Thus it is evident that the eosin used by Mallory when he first worked out his eosin-methylene-blue technic must have been a phloxine or some closely related dye rather than a true eosin. There is evidence that erythrosin B and phloxine have been put on the market labeled eosin bluish; so has a blend of eosin Y shaded with some one of the higher members of the group. Phloxine has been sold as magdala red and is even now sometimes marketed under that name both by American and foreign concerns. All this has caused much confusion; but dealers in stains are using greater caution in the matter at present.

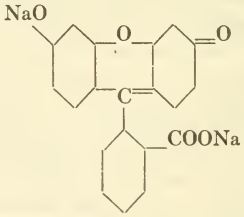
R1 FLUORESCEIN C. I. NO. 766

This is the simplest of the fluoran dyes, and is the mother substance of the eosins. Its sodium salt is known as *uranin*.



$C_{20}H_{12}O_5$; *Mol. Wt.* 332.296

Fluorescein



$C_{20}H_{10}O_5Na_2$; *Mol. Wt.* 376.274

Uranin

(*An acid dye: absorption maximum about 490*)

Solubility at 26°C:

Of fluorescein: in water, 0.03%; in alcohol, 2.21%.

Of uranin: in water, 50.02%; in alcohol, 7.19%.

Fluorescein is a yellow dye of very low tinctorial power, and hence of no value for ordinary staining purposes. It is on the other hand extremely fluorescent, the greenish yellow fluorescence being detectable in extremely high dilution. On account of this latter property the dye is used to determine the possibility of contamination from some suspected source getting into a neighboring water supply.

This fluorescence is even more pronounced in ultraviolet light, a fact which makes the dye useful in fluorescence microscopy. Thus Hercik (1939) employs it as a fluorochrome in the study of epidermis cells of *Allium;* Levaditi *et al.* (1940) in virus studies; Metcalf and Patton (1944) in insect entomology.

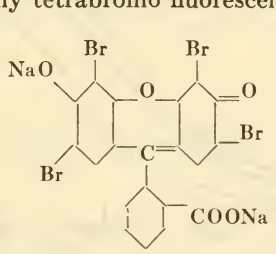
It has been included by Williams and Green (1935) in a series of dyes that prove useful in media in which fungi are growing, the absorption of the dye by the fungi giving characteristic colors to the colonies.

R5 EOSIN Y (i.e., YELLOWISH) C. I. NO. 768

Synonyms: *Eosin, water soluble. Bromo acid, J, TS, XL or XX. Bromofluorescein. Bronze bromo ES.*

Various shades denoted: *Eosin B extra, BP, BS, DH, G, GGF, J extra, 3J, 4J, JJF, KS, S extra, Y extra and YS.*

This dye is typically tetrabromo fluorescein:



$C_{20}H_6O_5Br_4Na_2$; *Mol. Wt.* 691.906

(*An acid dye; absorption maximum 515–518*)

Solubility at 26°C: in water 44.20%; in alcohol 2.18%

but the mono- and dibromo derivatives are also known and frequently occur in eosin. This affects the shade, as the more bromine present the redder the dye. It is plain that various mixtures of these compounds are on the market; but it has not yet been determined which are more suitable for biological purposes. Considerably more work on eosin is needed than has been done at the present time. From the name "water soluble eosin" it is often

assumed that this dye is not soluble in alcohol. This is not true, however.

A typical absorption curve of eosin Y is given in graph 2, Fig. 24, in which it is compared with the similar spectral data for eosin B and ethyl eosin.

Like fluorescein, eosin Y shows greenish yellow fluorescence, especially in alcoholic solution. This fluorescence is visible in

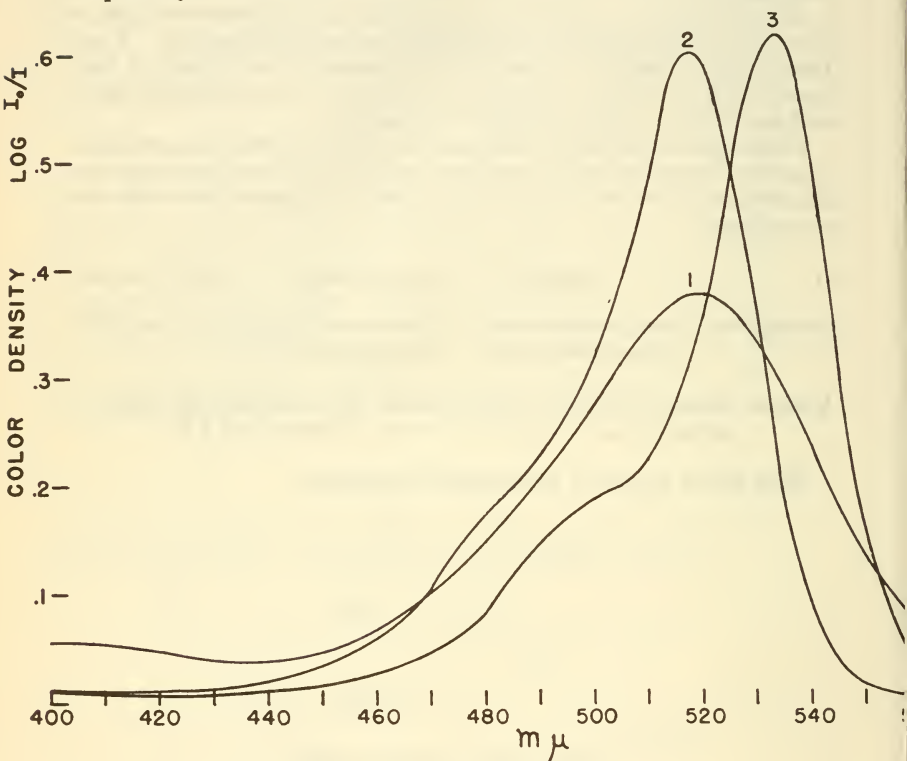


Fig. 24. Spectral curves of three eosins:
1. Eosin B. 2. Eosin Y. 3. Ethyl eosin.

ordinary light, but although quite strong, is not so pronounced nor visible in such great dilution as in the case of fluorescein. It is interesting that in this series of fluoran derivatives, tinctorial power increases but fluorescence decreases with the ascending members of the series; thus the erythrosins are less fluorescent than eosin, while phloxine and rose bengal show scarcely any fluorescence in ordinary light. In spite of the marked fluorescence of eosin Y, it does not seem to have been suggested as a fluorochrome.

Yellowish eosin is one of the most valuable plasma stains known. It is used in various technics for staining the oxyphile granules of cells (i.e., the granules having special affinity for acid dyes); these cell elements, in fact, being often called eosinophile granules because their presence was first recognized through the use of this dye. It is often employed as a counterstain for hematoxylin and the green or blue basic dyes; as for example by List with methyl green. Its uses, however, are really too numerous to list. At the present time one of the uses for which it is in greatest demand is as a blood stain in the technic of Romanovsky, with its various modifications, in which it is combined with methylene blue to form a "neutral" stain.

A dye mixture sold under the name of "Triosin", was described by Galigher (1934), and sold by him without stating its composition. The mixture proves valuable as a counterstain in some procedures where eosin Y alone gives less satisfactory results. Recently Emery and Stotz (1952) used this product as an unknown in a test of filter paper chromatography for dye analysis. In the course of this investigation the composition of the mixture became evident, and was stated by these authors as being apparently 62% eosin Y, 28% orange G, and 10% erythrosin Y. A spectral curve of the mixture, however, does not reveal the presence of either of the two latter dyes, but seems practically identical with that of eosin Y alone.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIS STAIN IS USED*

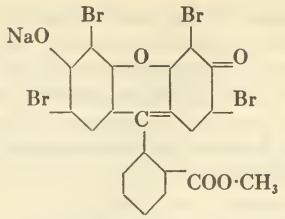
NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Delafield's hematoxylin with eosin Y	IA_4-11
Mayer's acid hemalum with eosin Y, Lillie modification	IA_4-13
Thiazin eosinates in tissue staining	IA_4-17
Buffered Mann stain	IA_4-19
Wright stain for blood films	ID_3-3
MacNeal's tetrachrome stain for blood films	ID_3-5
Giemsa stain for blood films	ID_3-6
Wright, Giemsa, or May-Gruenwald stain for bone marrow	ID_3-10-11
Counterstain in Gram-Weigert technic for bacteria in tissues	$IIIB_3$-8

*Under this heading are given references to procedures described in detail in STAINING PROCEDURES, edited by Conn and Darrow (1943-4).

R8 METHYL EOSIN C. I. NO. 769

Synonym: *Eosin, alcohol soluble.*

This is the methyl ester of yellowish eosin, the sodium salt of which is:



$C_{21}H_9O_5Br_4Na$; *Mol. Wt.* 683.943

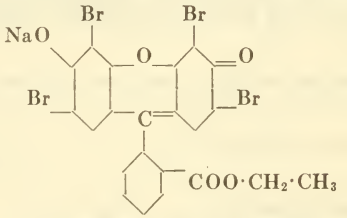
(*An acid dye; absorption maxima about 520*, [*485.5*])

There is no evidence that this dye has been furnished to biologists as a stain. The alcohol soluble eosin known in the biological laboratory is apparently the following.

R10 ETHYL EOSIN C. I. NO. 770

Synonyms: *Eosin, alcohol soluble. Eosin S.*

This is similar to methyl eosin, but is the ethyl ester:



$C_{22}H_{11}O_5Br_4Na$; *Mol. Wt.* 697.969

(*An acid dye; absorption maximum 524–527*)

Solubility at 26°C: in water 0.03%; in alcohol 1.13%

A spectral curve of this dye is given in graph 3, Fig. 24.

Ethyl eosin is apparently the alcohol soluble eosin ordinarily sold by biological supply houses. For some time after the first World War it was difficult to secure this dye in America, and one stain company for a few years furnished the color acid of eosin Y when alcohol soluble eosin was ordered. (The color acids of all these dyes are alcohol soluble but almost insoluble in either hot or cold water; methyl and ethyl eosin, on the other hand, are slightly soluble in cold water but strongly soluble in hot water and alcohol.) This error has now been corrected, and true ethyl eosin as obtainable from American manufacturers, is on the Commission

certification basis, and can be ordered from any biological supply house. It should be ordered as ethyl eosin rather than alcohol soluble eosin, as the latter name is less definite.

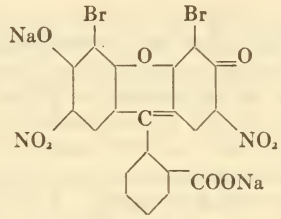
Ethyl eosin is a valuable counterstain after Delafield's hematoxylin. Preceding methylene blue, it is employed in demonstrating Negri bodies in the central nervous system of rabid animals.

For technic of **Harris stain for Negri bodies**, see Staining Procedures, p. IIIA$_2$-20.
For technic of **staining Negri bodies in sections**, see Id. p. IIIB$_3$-12, 13.

R15 **EOSIN B** (i.e. BLUISH) C. I. NO. 771

Synonyms: *Eosin BN, BA, BS, BW,* or *DHV. Saffrosin. Eosin scarlet. Eosin scarlet B. Scarlet J, JJ, V. Nopalin G. Imperial red.*

This is a dibrom derivative of dinitro fluorescein:



$C_{20}H_6N_2O_9Br_2Na_2$; *Mol. Wt.* 624.090

(An acid dye; absorption maximum 516–519)
Solubility at 26°C: in water 39.11%; in alcohol 0.75%

This dye has a distinctly bluer shade than eosin Y; although its absorption maximum is not very different, it has a distinctly different spectral curve, as shown by Fig. 24, p. 188.

It has occasionally been called for as a counterstain in some histological procedure as, for example, in aqueous solution following Mayer's hemalum (Kornhauser, 1930), but is not in general very valuable. Lillie (1944c) finds it specially useful, as a neutral stain, for tissues, as an azure A or toluidine blue eosinate.

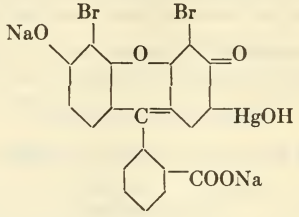
Ordinarily, if a shade deeper than eosin Y is desired, better results can be obtained with erythrosin, phloxine or rose bengal than with eosin B. Coleman and Bell, in fact, has put on the market a product labelled "eosin, bluish blend," which is a mixture of eosin Y with some one of the dyes just named; it is very satisfactory for certain staining procedures, but must not be confused with true eosin B.

For technic of **Mayer's hemalum with eosin B**, see Staining Procedures, p. IA$_4$-12.

For technic of **buffered azure eosinate method**, see Staining Procedures, p. IA$_4$-12.

R20 MERCUROCHROME 220

This is a proprietary name applied to a fluorescein derivative closely related to eosin: dibromo-hydroxy-mercuri-fluorescein.



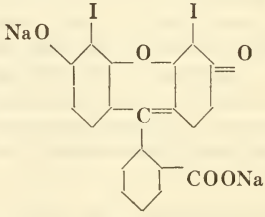
$C_{20}H_8O_6HgBr_2Na_2$; *Mol. Wt.* 750.700

The dye has been widely advertised as a disinfectant especially for the skin; and there has been considerable discussion in the literature as to its actual value for the purpose. It has staining properties not unlike an erythrosin, or phloxine. Baldwin (1928) states that it may be used in 2% aqueous solution in place of eosin, especially for blood work and for tissues after Zenker fixation; thus employed it is more intense than eosin and has a stronger affinity for cytoplasmic structures. Detwiler and McKennon (1929) employ it in concentration of from 1:500,000 to 1:1,000,000 as a fungicidal agent for the treatment of amphibian embryos. Conklin (1934) recommends it as a counterstain to malachite green in the Wirtz spore stain for bacteria.

R25 ERYTHROSIN, YELLOWISH C. I. NO. 772

Synonyms: *Erythrosin R or G. Pyrosin J. Dianthine G. Iod-eosin G.*

This is a fluorescein in which there are two substituent iodine atoms instead of four bromine atoms as in yellowish eosin.



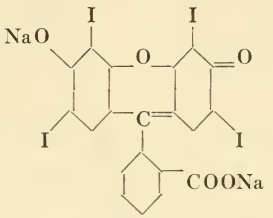
$C_{20}H_8O_5I_2Na_2$; *Mol. Wt.* 628.098

(*An acid dye; absorption maximum about 510.5*)

R26 ERYTHROSIN, BLUISH C. I. NO. 773

Synonyms: *Erythrosin B, N,* or *JN. Pyrosin B. Eosin J. Iodeosin B. Dianthine B.*

This is the tetraiodo compound corresponding to the tetrabrom compound of typical eosin.



$C_{20}H_6O_5I_4Na_2$; *Mol. Wt.* 879.922

(*An acid dye; absorption maximum 524–527*)

Solubility at 26°C: in water 11.10%; in alcohol 1.87%

There is some uncertainty as to which of these two very similar dyes is preferable in any given technic. At present, the latter, erythrosin B, is ordinarily furnished by supply houses when "erythrosin" is ordered. Its spectral curve is given in Fig. 25, p. 194.

Erythrosin has some use as an indicator. It is also employed as a contrast stain for hematoxylin and certain blue and violet nuclear stains. Thus, for example, Held (1895) used it, preceding methylene blue, as a plasma stain for nerve cells. Similarly the technic of Jackson (1926) calls for it as a counterstain to crystal violet in plant histology. It was employed by Winogradsky (1924) for staining bacteria in soil. Gellhorn (1931) applied it to the vital staining of sea urchin eggs. These procedures are mentioned merely by way of illustration; the uses of erythrosin are quite numerous, and the list could be extended almost indefinitely. For these purposes probably the tetra-iodo compound (i. e., erythrosin bluish) is desired; but the literature is vague on the subject.

A sample of erythrosin of pre-war origin that was labeled magdala red has been examined by the Commission. This mislabeling undoubtedly explains Chamberlain's results already mentioned (page 133) in staining algae. Chamberlain, it will be recalled, was able to obtain good results with a low-priced product called magdala red but not with the high-priced stain called magdala red *echt.*

For technic of **Jackson stain for woody tissue,** see Staining Procedures, p. IIA-9.

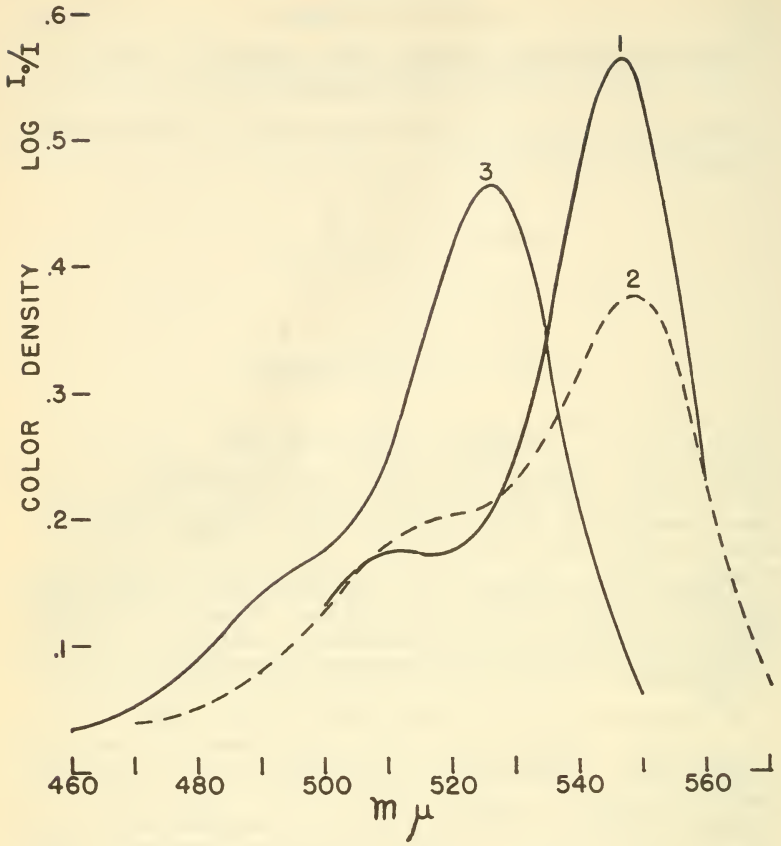


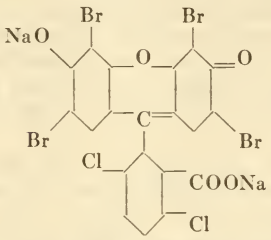
FIG. 25. Spectral curves of three xanthene dyes:

1. Phloxine B. 2. Rose Bengal. 3. Erythrosin B.

If in the manufacture of fluorescein, dichloro or tetrachloro phthalic acid is used for condensation with resorcin, a series of halogenated derivatives may be obtained differing from those just named by having halogen atoms in the phthalic acid residue of the molecule as well as in the resorcin residue. These compounds are slightly deeper in shade than the corresponding derivatives of simple phthalic acid; and they are generally regarded as being of more pleasing shade than the ordinary fluorescein dyes. The phloxines and rose bengals are the best known of these dyes.

R30 PHLOXINE C. I. NO. 774

Synonyms: *Erythrosin BB, or B extra. New pink.*



$C_{20}H_4O_5Br_4Cl_2Na_2$; *Mol. Wt.*.760.804

(*An acid dye; absorption maxima about 535.7, [497.1]*)
Solubility at 26°C: in water 50.9%; in alcohol 9.02%

This dye and the following are often denoted interchangeably phloxine or phloxine B, and there is some question as to whether biologists have always been furnished the same type. The phloxines of American manufacture at present on the market are the following type.

R31 PHLOXINE B C. I. NO. 778

Synonyms: *Phloxine TA, N, BP super, RB, TB, or BB. Cyanosine. Eosin 10B.*

$C_{20}H_2O_5Br_4Cl_4Na_2$; *Mol. Wt.* 829.702

(*An acid dye; absorption maxima 546–548*)

This dye differs from C. I. No. 774 in having four instead of two chlorine atoms in the phthalic acid residue of the molecule, Samples of phloxine recently submitted to the Commission for certification seem to vary in degree of bromination or chlorination, but correspond to this C. I. number rather than to the preceding. Although some of the procedures given below may have been worked out originally with the other type of phloxine, this type has been found to give satisfactory results.

A spectral curve of this dye, in comparison with erythrosin B and rose Bengal, is given in Fig. 25.

Unna (1921) used phloxine in combination with several other acid dyes in studying the process of chromolysis. The dye was seldom specified for biological work until about 1925, but has recently been applied to staining bacteria, Negri bodies, and to various histological procedures. It has, moreover, frequently been used under other names.

Chamberlain (1932, page 69; 1927), mentions having used it successfully in place of magdala red in staining algae. His original technic called for magdala red; but true magdala red does not serve his purposes. Inasmuch as erythrosin (see above) was evidently sold in the past as magdala red and Chamberlain can duplicate his original results with phloxine, the chances are that some of the magdala red formerly available was either phloxine or else that phloxine and erythrosin give similar results by Dr. Chamberlain's technic.

About 1920 Dr. F. B. Mallory, like many others, found it difficult to obtain an eosin of either domestic or foreign origin which would give consistently reliable results by his eosin-methylene blue method described in 1904. After testing out, on the advice of a member of the Commission, a series of eosins and closely related dyes he wrote that phloxine is "the best *eosin* I have yet found for use in the eosin-methylene blue stain for paraffin sections of tissues fixed in Zenker's fluid."* Here again is a case where phloxine apparently was obtained before the war under an incorrect name and the incorrect name used in the publication of a well-known technic.

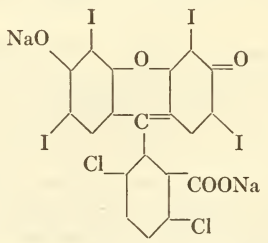
For technic of **Mallory's phloxine-methylene-blue stain**, see Staining Procedures, pp. IA_4-20; $IIIB_3$-4.

For use as **counterstain in Gram-Weigert technic for bacteria in tissues**, see Id. p. $IIIB_3$-8.

B35 ROSE BENGAL G C. I. NO. 777

Synonym: *Rose SA.*

Various shades denoted: *Rose bengal N, AT, NT, NTO, and B.*



$C_{20}H_4O_5I_4Cl_2Na_2$; *Mol. Wt.* 948.820

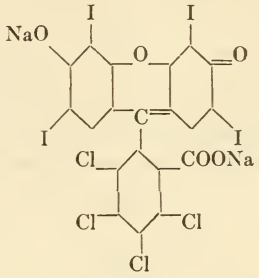
(*An acid dye*)

This particular rose bengal apparently has not been employed by biologists. The dye of that name sold by stain companies at present is the following.

*Quoted from personal letter.

R36 ROSE BENGAL C. I. NO. 779

Various shades denoted: *Rose bengal extra, 3B Conc., N extra, DY, B* and *2B*.



$C_{20}H_2O_5I_4Cl_4Na_2$; *Mol. Wt.* 1017.718

(*An acid dye; absorption maximum 544–548*)

Solubility at 26°C: in water 36.25%; in alcohol 7.53%

The dye has a pleasing deep pink color; and although an acid dye it proves to have considerable affinity for bacterial protoplasm, and to have good selective properties when used as a bacterial stain. It has been recommended (Conn 1918, 1921) for staining bacteria, especially in soil suspensions. It has also been used as a cytoplasm stain following hematoxylin or preceding toluidine blue. Maneval (1934) employed it in the negative staining of bacteria and Ono (1934) for spirochaetes in blood. It also finds use in Delprat and Stowe's test (1931) for liver function. Popper (see Metcalf and Patton, 1944) mentions it as a useful fluorochrome in the study of fats under ultraviolet illumination. Smith and Dawson (1944) employ this dye as a bacteriostatic agent in media designed to permit the growth of soil fungi while repressing the bacteria; this is the only instance yet noted of one of the fluoran derivatives being thus used.

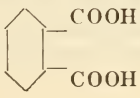
From Fig. 25, in which the spectral curve of this dye and phloxine B are compared, it will be seen that they differ more in shape than in the position of the absorption maximum.

For technic of **general staining of pure cultures of bacteria**, see Staining Procedures, p. $IIIA_2$-5.

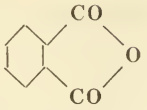
For technic of **Conn's stain for bacteria in soil**, see Staining Procedures, p.$IIIA_2$-21.

4. PHENOLPHTHALEIN AND THE SULFONPHTHALEINS

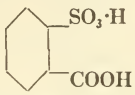
A phthalein is a compound of phthalic acid:



or rather of phthalic anhydride:

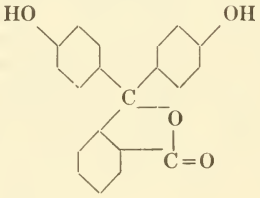


with phenol or a phenol derivative. If phthalic acid is heated with phenol and sulfuric acid it combines with two molecules of the phenol and forms phenolphthalein. In the same way, a sulfonphthalein is a compound of ortho-sulfo-benzoic acid:

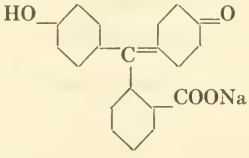


and phenol or a phenol derivative. These compounds, although sometimes behaving as dyes, are not used as dyes or stains, but as indicators. For this purpose the members of the group are very valuable.

Phenolphthalein, although not used as a dye, is colored and is apparently capable of salt formation. In acid solutions it is colorless, and is assumed to have the formula:



Upon neutralization the alkali is believed to attach itself to the CO-group, which breaks the five-sided ring (the lactone ring) and causes one of the benzene rings to take on quinoid form, thus:



With this change, the red color of the compound appears, but disappears again if the solution is made acid so as to destroy the quinoid structure. This makes the compound a very valuable indicator.

The dyes of this group have no value in practical dyeing, although they are all weakly acid dyes. Similarly they are of little use as biological stains, except occasionally in vital staining. Their real value is as acid-base indicators. Even when they are employed as vital stains, they serve primarily to study the reaction of tissues or body fluids. They are also valuable indicators in micro-injection of cells.

Hydrogen-ion indicators. The sulphonphthaleins, together with phenolphthalein, are among the most useful indicators known to the chemist. Quite a long series of them has been prepared, which in general show their deepest color in alkaline solutions and turn yellow on the addition of acid. Some of them, such as thymol-sulfonphthalein (thymol blue), show two colors besides yellow, one in strong acid solutions and the other in strong basic solutions, while in solutions near the neutral point they are yellow. That these color changes are due to alterations in the structure of the molecule, such as the disappearance and reappearance of the quinoid ring, is generally assumed; but in the case of these compounds the relation of structure to color is complicated and has not yet been worked out to general satisfaction.

The colorimetric measurement of H-ion concentration depends upon these color changes. The color change of an indicator takes place within a short range on each side of that point in the H-ion scale at which the dye is 50% dissociated, i.e., occurs half in the form of the undissociated dye and half in the form of free ions. This point on the pH-scale (at which an indicator is 50% dissociated) is important, and corresponds to the invert logarithm of its dissociation constant. The dissociation constant of an indicator, in other words, is a quantity whose invert logarithm is the pH-value of the point at which the indicator is half dissociated. The approximate invert logarithm of the apparent dissociation constant is denoted by chemists by the symbol pK; the value of this quantity is given in the case of each of the indicators listed below.

For a short distance on each side of the dissociation constant, every shade of the indicator corresponds to a definite pH-value; this zone is known as the sensitive range of the indicator. Roughly speaking, the sensitive range of any indicator extends for about 0.8 pH to each side of its pK-value. Throughout its sensitive range, an indicator can be used to determine the H-ion concentration of a solution by comparing its shade with that produced in standards of known reaction. The various indicators of this group differ in their strengths as acids (i. e. the extent to which they dissociate); and the greater their strength the lower the pH-value of the point at which they are 50% dissociated. It is plain, therefore that different indicators may be selected according to their dissociation constants to cover successive portions of the H-ion scale.

Fortunately nearly all of them have different dissociation constants; so that by employing a series of them one may determine the H-ion concentration of solutions of any reaction ordinarily encountered.

There are two general types of indicators, those which show only one color in the useful range, and those which change from one color to another. The phthaleins, such as phenolphthalein, change from colorless substances in their acid ranges to colored compounds in their alkaline ranges. The sulfonphthaleins, on the other hand, change from yellow to some deeper color. With the first

TABLE 3

COLOR CHANGES OF THE INDICATORS OF CLARK AND LUBS AND OF COHEN

INDICATOR	MOLECULAR WEIGHT	CONCENTRATION RECOMMENDED*	FULL ACID COLOR	FULL ALKALINE COLOR	SENSITIVE RANGE pH	pK†
Meta-cresol purple (acid range)	382.414	0.04	Red	Yellow	1.2–2.8	1.5
Thymol blue (acid range)	466.570	0.04	Red	Yellow	1.2–2.8	1.5
Brom phenol blue	669.994	0.04	Yellow	Blue	3.0–4.6	4.1
Brom chlor phenol blue	581.076	0.04	Yellow	Blue	3.0–4.6	4.0
Brom cresol green	698.046	0.04	Yellow	Blue	3.8–5.4	4.7
Chlor cresol green	520.210	0.04	Yellow	Blue	4.0–5.6	4.8
Methyl red	269.294	0.02	Red	Yellow	4.4–6.0	5.1
Chlor phenol red	423.260	0.04	Yellow	Red	4.8–6.4	6.0
Brom phenol red	512.178	0.04	Yellow	Red	5.2–6.8	6.2
Brom cresol purple	540.230	0.04	Yellow	Purple	5.2–6.8	6.3
Brom thymol blue	624.386	0.04	Yellow	Blue	6.0–7.6	7.0
Phenol red	354.362	0.02	Yellow	Red	6.8–8.4	7.9
Cresol red	382.414	0.02	Yellow	Red	7.2–8.8	8.3
Meta cresol purple (alkaline range)	382.414	0.04	Yellow	Purple	7.4–9.0	8.3
Thymol blue (alkaline range)	466.570	0.04	Yellow	Blue	8.0–9.6	8.9
Cresolphthalein	346.364	0.04	Colorless	Red	8.2–9.8	9.4
Phenolphthalein	318.312	0.04	Colorless	Red	8.3–10.0	9.7

*In 95% ethyl alcohol.

†Approximate invert logarithm of apparent dissociation constant.

type of indicator the H-ion concentration may be determined by the alteration in intensity of color, with the second type by alteration in hue. The two-color indicators, such as the sulfonphthaleins, are more satisfactory because one can measure changes in hue more accurately than changes in color intensity, especially when one must depend on rather crude methods of colorimetry as is usually the case when indicators are employed.

The sulfonphthalein indicators are especially valuable as indicators for still other reasons. Unlike the azo compounds, such as methyl red, they are very stable chemically, while they are less

affected by the presence of neutral salts and proteins than are many other indicators.

The first list of sulfonphthalein indicators was published by Clark and Lubs (1917), who to make their series complete had to include methyl red, an azo dye, less satisfactory because its solutions are subject to reduction on standing. More recently Cohen (1923, 1926) added six new sulfonphthalein indicators, among them two (brom cresol green and chlor cresol green) which have sensitive ranges so close to methyl red as to make the latter unnecessary.

These indicators, unlike the usual dyes, are ordinarily furnished in the form of color acids, which are practically insoluble in water. They must accordingly be employed in alcoholic solution or must be converted into the di-sodium salt with the proper amount of NaOH, the quantity of the latter to use being figured by the following formula:

$$\text{Weight NaOH} = \frac{\text{Weight indicator} \times 40}{\text{Molecular weight indicator}}.$$

The alcoholic solutions are simpler to prepare and for ordinary indicator purposes are equally satisfactory; they cannot, of course, be employed, in vital staining.

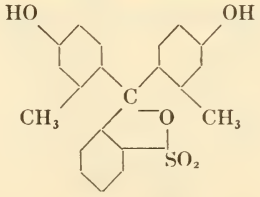
Table 3, above, quoted from Leaflet IX of the Manual of Methods for Pure Culture Study of Bacteria (Soc. of American Bacteriologists; published by Biotech Publications, Geneva, N. Y.), shows the color changes and sensitive ranges of the indicators described below. (This table also includes methyl red, which is an azo dye, and is described in Chapter V of this book.)

In the following list the compounds are arranged in the order of their dissociation constants and hence in that of their sensitive ranges in the pH-scale.

s5 META-CRESOL PURPLE

$$pK = 1.5,\ 8.3$$

This indicator is *m*-cresol sulfonphthalein.

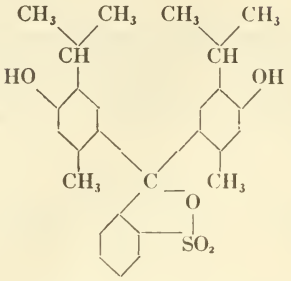


An indicator changing from red to yellow in its acid range and from yellow to purple in its alkaline range.

s10 THYMOL BLUE

pK = 1.5, 8.9

This is thymol sulfonphthalein:

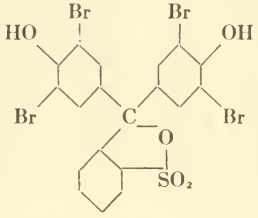


Thymol blue is now a well known indicator, used both in its acid range (red to yellow) and in its alkaline range (yellow to blue). In the latter range it may well be replaced with meta-cresol purple.

s15 BROM PHENOL BLUE

pK = 4.0

This is tetra-bromo-phenol sulfonphthalein.

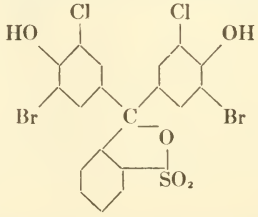


An indicator changing from yellow to blue.

s20 BROM CHLOR PHENOL BLUE

pK = 4.0

This is di-bromo-di-chloro-phenol sulfonphthalein.

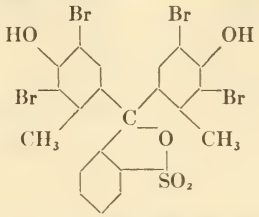


An indicator almost identical with the last in range, and showing the same change in color.

s25 BROM CRESOL GREEN

pK = 4.7

This is tetra-bromo-meta-cresol sulfonphthalein:

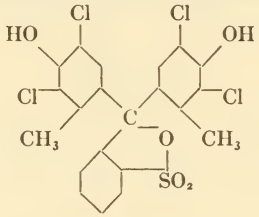


An indicator changing from yellow to blue.

s30 CHLOR CRESOL GREEN

pK = 4.8

This is tetra-chloro-meta-cresol sulfonphthalein.

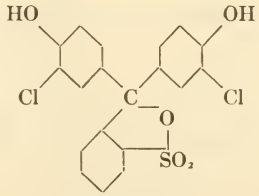


An indicator changing from yellow to blue through a range only a little more acid than that of methyl red.

s35 CHLOR PHENOL RED

pK = 6.0

This is di-chloro-phenol sulfonphthalein.

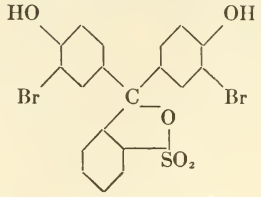


An indicator changing from yellow to red.

s40 BROM PHENOL RED

pK = 6.2

This is di-bromo-phenol sulfonphthalein.

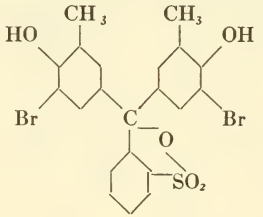


An indicator changing from yellow to red through a range almost identical with that of the better known brom cresol purple. It is recommended in place of the latter because it is free from troublesome dichromatism.

45 BROM CRESOL PURPLE

pK = 6.3

This is di-bromo-ortho-cresol sulfonphthalein:

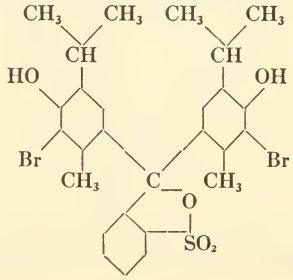


A well-known indicator changing from yellow to purple. It has been used by McMaster and Parsons (1938) as a vital stain.

s50 BROM THYMOL BLUE

pK = 7.0

This is di-bromo-thymol sulfonphthalein:

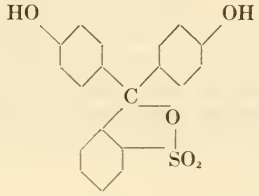


It is a very valuable indicator because of its dissociation constant at close to true neutrality. It is yellow in acid, green in neutral, and blue in alkaline solutions. It has also been employed as a vital dye by McMaster and Parsons (1938), and as a stain for fungi in roots by Garrett (1937).

s55 PHENOL RED

pK = 7.9

This is phenol-sulfonphthalein:

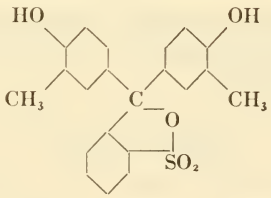


An indicator changing from yellow to red, employed for some time in physiological work, and well known before the paper by Clark and Lubs above mentioned. The range of this indicator (approx. pH 6.8–8.4) makes it quite valuable in much biological work; it is quite commonly used in the study of kidney function.

s60 CRESOL RED

pK = 8.3

This is ortho-cresol-sulfonphthalein:

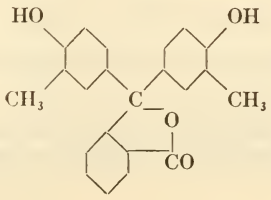


An indicator changing from yellow to red

s65 CRESOLPHTHALEIN

pK = 9.4

This indicator is closely related to phenolphthalein, having the constitution:



It is a newer indicator than phenolphthalein, and is generally regarded as preferable when used in conjunction with the sulfonphthaleins, because its range does not overlap quite so far the alkaline range of thymol blue. It is a one-color indicator and its color change is the same as that of phenolphthalein.

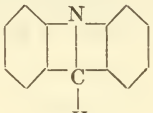
s70 PHENOLPHTHALEIN

$pK = 9.7$

This indicator, of which the formula is given on p. 198, is quite useful in the alkaline range centering around pH 9.7, with a color change from red in alkaline solutions to colorless in acid ones. It has the disadvantage of a one-color indicator discussed on p. 200.

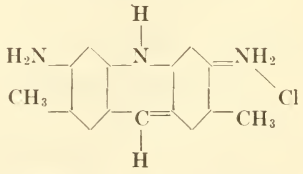
5. ACRIDINE DYES

There is a small group of dyes derived from the compound acridine



which is closely related to xanthene. They are not xanthene dyes, but are included in this chapter for convenience. There are a few of interest to the biologist.

t5 ACRIDINE YELLOW C. I. NO. 785

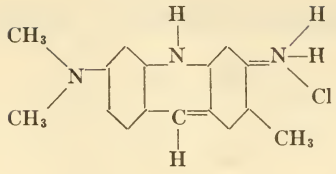


$C_{15}H_{16}N_3Cl$; *Mol. Wt.* 273.759

[*A basic dye; absorption maximum about 455 (Formánek)*]

This dye is rarely called for in microtechnic, but has been referred to by Metcalf and Patton (1944) as a fluorochrome useful in insect histology.

T10 CORIPHOSPHINE O C. I. NO. 787

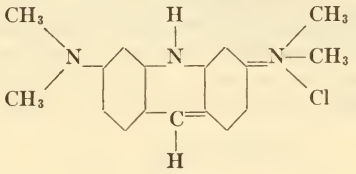


$C_{16}H_{18}N_3Cl$; *Mol. Wt.* 287.785

[*A basic dye; absorption maxima about 643, 482 (Formánek)*]

T15 ACRIDINE ORANGE NO C. I. NO. 788

Synonyms: *Basic orange 3RN. Euchrysine 3RXA.*

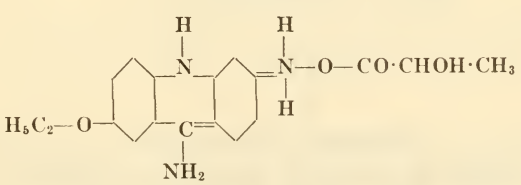


$C_{17}H_{20}N_3Cl$; *Mol. Wt.* 301.811

[*A basic dye, ordinarily obtained as the zinc chloride double salt; absorption maxima about (467) 497 (Formánek)*]

Both of these dyes are among those employed as fluorochromes by Metcalf and Patton (1944). Strugger (1948) has used acridine orange for demonstrating bacteria in soil suspensions, by means of ultraviolet light; bacteria are green, humus particles red. Their chief use, in fact, is as fluorochromes; but acridine orange has recently attracted some attention because it stains tumor cells selectively, intra vitam, and causes retardation of tumor growth (Lewis and Goland, 1948).

T20 RIVANOL

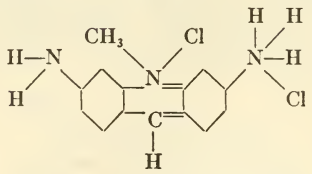


$C_{18}H_{21}N_3O_4$; *Mol. Wt.* 343.372

This has also been called for by the two authors above mentioned, as a fluorochrome.

T25 ACRIFLAVINE C. I. NO. 790

Synonym: *Trypaflavine. Flavine.*



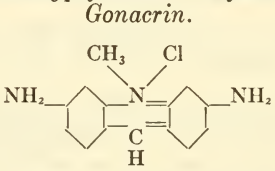
$C_{14}H_{15}N_3Cl_2$; *Mol. Wt.* 296.198

(*A basic dye*)

This yellow dye is one of those developed by Ehrlich for its therapeutic value. It is marketed at present as a disinfectant. It is employed as a bacteriostatic agent by Churchman (1927) mixed with methyl and crystal violets under the name of "acriviolet." Acriflavine has little use as an ordinary stain, but is coming to be employed to some extent as a fluorochrome; see Levaditti *et al.* (1940), Metcalf and Patton (1944).

T30 NEUTRAL ACRIFLAVINE

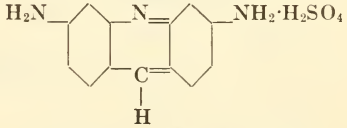
Synonyms: *Neutral trypaflavine. Euflavine. Neutroflavine. Gonacrin.*



$C_{14}H_{14}N_3Cl$; *Mol. Wt.* 259.733

This dye finds use as a disinfectant. It is said to be less irritating than acriflavine, because it is neutral.

T35 PROFLAVINE C. I. NO. 790



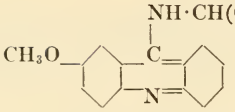
$C_{13}H_{12}N_3SO_4$; *Mol. Wt.* 306.310

(*A basic dye ordinarily supplied as the sulfate*)

Like the very similar acriflavine, this is used primarily as a disinfectant. It has been called for by Rogers (1940) as an agent for inhibiting sulfate-reducing bacteria.

T40 ATABRINE

Synonym: *Atebrine* (in Germany).



$C_{23}H_{31}N_3O$; *Mol. Wt.* 365.502

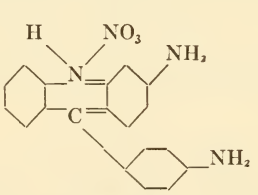
This is a yellow compound used primarily for its medicinal value (as an antimalarial agent). It shows fluorescence in ultraviolet light, and has been called for by Metcalf and Patton (1944) as a fluorochrome in insect histology.

Atebrine and a few other acridine antimalarials are among the dyes which Lewis and Goland (1948) have found to stain and to retard tumors in mice.

T45 PHOSPHINE C. I. NO. 793

Synonyms: *Leather yellow. Xanthin.*

A mixture of nitrates of chrysanilin, and probably some of its higher homologs, chrysanilin having the following formula:



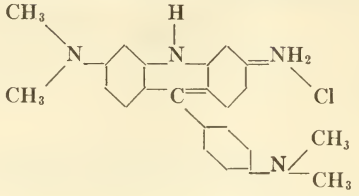
$C_{19}H_{10}N_2O_2$; *Mol. Wt.* 348.350

(*A basic dye; principal absorption in the neighborhood of 480–490*)

This dye has been used (in 1% solution) by Schumacher (1922) following methylene blue (1% phenolic solution) to stain bacterial nucleins and nucleoproteids yellow while the free or unbound nucleic acid is green. Metcalf and Patton (1944) employ phosphine 3R, one of the redder shades of this dye, as a fluorochrome in the study of insect histology by fluorescence microscopy.

T50 RHEONINE A C. I. NO. 795

Synonyms: *Rheonine AL, G or N. Fast phosphine NAL.*



(*A basic dye; absorption maximum about 464.5*)

Although seldom called for in biological work, this dye is mentioned by Pick (1935) as a fluorochrome useful in vital staining of the frog.

CHAPTER IX
NATURAL DYES

THE NATURAL DYES

AS STATED in Chapter II the group of natural dyes is shrinking, as more and more of them are being produced by artificial means. Alizarin, for example, in the form of madder, used to be extracted from the roots of *Rubia tinctorum;* but the artificial manufacture of this dye is now much more economical. The group of natural dyes, as ordinarily recognized, contains only those which are not yet produced by artificial means. Indigo, however, is listed in this chapter, because in its chemistry it does not fall in well with any of the groups of artificial dyes. It is still obtained in part, moreover, from the indigo plant, although under present-day conditions its artificial manufacture is ordinarily the more economical. Similarly, orcein can now be synthetically prepared, but is still included in this chapter because of its relationship to other dyes that are secured from natural sources only.

The chemistry of the natural dyes is less definitely known than that of the artificial dyes. This is easily understood; for it will be recalled that there are two ways of obtaining information as to the chemistry of unknown compounds: the first by decomposing them into simpler compounds of known composition; and the second by manufacturing them from known compounds. In the case of dyes not yet prepared artificially the second of these two lines of procedure is out of the question; hence there is special difficulty in learning their exact chemical structure.

The most important natural dyes for the biologist are hematoxylin, indigo, cochineal (and its derivatives), orcein, and litmus.

U5 **SAFFRON**

A natural pigment of uncertain composition extracted from the stigmas of *Crocus sativus.* It has been employed by Masson (see Foot, 1933) in a connective tissue stain. A special application of this procedure has been made by Block and Godin (1936) for staining yellow fever lesions in the liver. A later application by Van Hoecke and Sebruyns (1952) calls for this dye, after Bismarck brown, aniline blue W.S. and hematein, as a differential stain for glandular cells of the stomach.

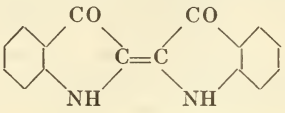
THE INDIGO GROUP

U10 **INDIGO** C. I. NO. 1177

Synonym: *Indigo blue.*

The plants from which indigo was formerly exclusively manu-

factured are largely species of the genus known as *Indigofera*, although some indigo-bearing plants are recognized by botanists as belonging to different genera. In these plants is a glucoside, indican, which is converted by fermentation into the dye indigo. Various formulae have been given for indigo; the one favored at present is based upon its method of artificial manufacture:



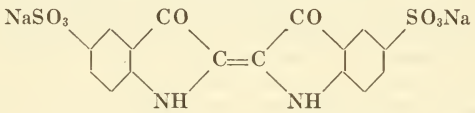
$C_{16}H_{10}N_2O_2$; *Mol. Wt.* 262.256

In this formula the exact chromophore group is uncertain; but the ketone group (CO) in a closed ring occurs so often in dyes that it is regarded as probably having chromophoric properties; the same is true of the C=C linkage.

U12 INDIGO-CARMINE C. I. NO. 1180

Synonym: *Indigotine Ia.*

This is the sodium salt of indigo disulfonic acid:



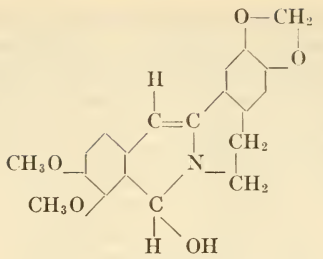
$C_{16}H_8N_2O_8S_2Na_2$; *Mol. Wt.* 466.354

Indigo carmine is a blue dye of acid properties, which is sometimes used as a plasma stain in contrast to some red dye such as carmine or basic fuchsin (see Shumway, 1926), sometimes hematoxylin. A few special applications of the stain can be mentioned: by Petragnani (1928), mixed with acid fuchsin, as a stain for Negri bodies; by Cuyler (1932), mixed with eosin Y, as a counterstain to hematoxylin for staining vaginal smears; by Brozek (see Hruby, 1933) in plant cytology where it is used in picric acid solution in contrast to basic fuchsin; by Kempton, Bott and Richards (1937) for uretral injection in studies of glomerular excretion.

For use in **Fraenkel's method for elastic tissue**, see Staining Procedures, p.IB$_3$-17.

U15 BERBERINE C. I. NO. 1237

Berberine was originally obtained from barberry and is sometimes called barberry extract. It is found in other plants, however, and can also be prepared synthetically. It has the following formula:



$C_{20}H_{19}NO_5$; *Mol. Wt.* 353.360

This compound acts as a basic dye. It has rarely been specified in biological work; but Metcalf and Patton (1944) mention it as a fluorochrome in connection with insect histology.

U20 COCHINEAL PRODUCTS C. I. NO. 1239

Cochineal is a dye that has long been well known. It is obtained from a tropical insect generally known as the cochineal insect. By grinding and extracting the dried bodies of the female of the species in question a deep red dye is obtained, which is known as cochineal. On treatment with alum this solution yields a product somewhat more free from extraneous matter, known as carmine. This is the form in which the dye is generally obtained by the microscopist. Cochineal products are used in various ways in microscopic technic, generally as nuclear dyes. They are extremely valuable in cases where it is desirable to stain in bulk before sectioning.

Cochineal, itself, has been used for various purposes in microscopic technic, even though less used today than carmine. Alone it has little value, to be sure, for it has no direct affinity for tissues unless they contain iron, aluminium or some other metal. It is most commonly employed either with or following a salt of one of these metals as a mordant. A tincture of cochineal, that is an alcoholic solution containing calcium and aluminium chlorides, has been used by Mayer (see Lee, 1937, p. 149) both on sections and for staining in bulk; but its most common method of use is with alum in watery solution. An alum-cochineal of this sort was first used independently by Mayer (1878, 1892) and Czokor (1880); it can be used for sections, and is specially recommended for staining in bulk, by which technic it stains nuclei violet red, and blood and muscle cells orange, while the cytoplasm is but weakly colored. A chrom-alum-cochineal has been used by Hansen (1905) for staining sections. Spuler (1901) recommends an iron-alum-cochineal for staining in bulk when the sections are to be photographed, the technic bringing out nuclei, the blood in the tissues, and the muscle striations; sections may also be stained by the same method. By

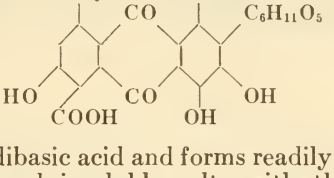
this technic the iron alum is applied first to the tissues as a mordant, and then followed by the stain. In Hansen's ferri-cochineal, on the other hand, the iron alum is mixed with the dye, and the mixture used for staining sections of tissue (see Lee, 1937, p. 145). Reynolds (1936) has proposed a mixture of alum cochineal with hematoxylin in staining trematodes and nematodes, *in toto*.

Carmine. Carmine is of considerable historic interest. It was used as early as 1770 by Hill and in 1838 by Ehrenberg, although as stated elsewhere not exactly for histological purposes. It was also employed in 1849 by Göppert and Cohn, by Corti in 1851, and by Hartig in 1854–8, these being the first uses of dyes in histology. It is still a valuable stain today, in spite of the enormous variety of synthetic dyes now available. It is much used for staining in bulk, particularly in embryological work. A well-known formula is Schneider's aceto-carmine (see Lee, 1937, p. 142), which is a valuable chromatin stain for fresh material in smear preparations. Belling's (1921) iron aceto-carmine is valuable for staining chromosomes in smear preparations from anthers. Alum carmine was used by Grenacher (1879) for similar purposes. Carmine is only slightly soluble in water at a neutral reaction; so solutions must be either acid (like the three above) or alkaline. Three alkaline formulae are of considerable use: ammonia carmine, which has been used both for injection and for staining sections; soda carmine, used primarily for injection; and Mayer's (1892, 1896) magnesia carmine, useful either for sections or for staining in bulk. Orth (1883) proposed a lithium carmine (i.e., dissolved in a solution of Li_2CO_3) as a nuclear stain for tissues; and the formula for this stain, as given by Krause (1926, I, p. 265), is still frequently employed. Alcoholic solutions are also used: Grenacher's borax carmine (or as modified by Mayer, 1892, 1896) being a splendid nuclear stain for sections; and the hydrochloric carmine of Mayer serving both for sections and for staining in bulk. A special formula containing aluminium chloride (known as muci-carmine) has been proposed by Mayer (1892, 1896) and is used for staining mucin. In double staining it is sometimes used with indigo carmine; but most often with picric acid or spirit blue. Picro-carmine is a very well-known combination used for double staining effects in sections, particularly for nervous tissue; it stains nuclei red and cytoplasm yellow.

One of the important uses of carmine is in Best's (1906) carmine stain for glycogen. The method is simple and the result beautiful, the red glycogen standing out in sharp contrast to the blue of the nuclei after staining in alum hematoxylin. The stain is permanent; the method is of much importance both to the pathologist and to the histologist.

Carminic acid. The dye principle of carmine and cochineal is carminic acid. This product is obtained by extracting the insect

bodies with boiling water, treating the extract with lead acetate or barium hydrate, and then decomposing the lead or barium carminate with sulfuric acid. The exact composition of carminic acid is still somewhat uncertain; so far as known, it is:



It is a fairly strong dibasic acid and forms readily soluble salts with the alkali metals, and insoluble salts with the heavy metals. Aluminium carminate (obtained by precipitation from aluminium acetate and carminic acid or ammonium carminate) is soluble in aqueous or weak alcoholic solutions of acids.

A slightly different aluminium compound, formed by mixing alum and carminic acid is used in histology. This combination was called carmalum by Mayer (1892, 1899), and has also been used by Grenacher (1879) and Rawitz (1899); it is a useful nuclear stain for sections; and is often employed with light green or indigo carmine as a contrast stain. A so-called muci-carmine, an acid solution containing aluminium chloride, has been employed by Rawitz to stain mucin; while Mayer's para-carmine, containing aluminium and calcium chlorides, is used both for sections and for staining in bulk. By others a combination of iron with carminic acid has been used for similar purposes.

Carmein. Carmine, kept in ammoniacal solution, changes in its properties, due to oxidation. The oxidized carmine, often known as carmein, can be obtained by treating a carmine solution with hydrogen peroxide and precipitating with alcohol. It is a dark colored mass which can be ground into a black powder.

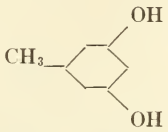
PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH COCHINEAL DERIVATIVES ARE USED.*

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Mayer's mucicarmine stain	IA_4-7
Mucicarmine with hematoxylin and metanil yellow	IA_4-7
As nuclear stain with Weigert's resorcin fuchsin for elastin	IB_3-13
Best's method for glycogen	ID_3-21
Grenacher's borax carmine in plant histology	IIA-5
Aceto-carmine and basic fuchsin for pollen tubes in style	IIA-13
Belling's iron-aceto-carmine for plant cytology	IIB-3
Aceto-carmine with chlorazol black E in plant cytology	IIB-4

*Under this heading are given references to procedures described in detail in Staining Procedures, edited by Conn and Darrow (1943-4).

Orcein and Litmus C. I. No. 1242

Both orcein and litmus are obtained from certain lichens, *Lecanora tinctoria* and *Rocella tinctoria*. These lichens are colorless, but when treated with ammonia and exposed to the air, blue or violet colors develop. The colors are due to certain phenolic compounds, one of which is orcinol:



Orcinol, acted upon by air and ammonia, becomes orcein.

U30 ORCEIN

The exact formula of orcein is unknown. It is a weak acid, soluble in alkalies, with a violet color. It is possible to synthesize this dye from orcinol by the above-indicated process. As a matter of fact very good synthetic products, for some purposes better than the natural product, are now on the market. The synthetic orceins from different manufacturers, however, are prepared by different methods and are not the same in their behavior. The reason for these differences are still unknown. The method of manufacture has never yielded exact information as to its structural formula; but it is believed to contain in its molecule the residues of four orcinol molecules. It has some indicator properties both for pH and oxidation-reduction; but is not as useful in that respect as its closely related litmus. It is a weak acid, violet in alkaline solution; in acid solutions it is orange-red.

Unna (see Lee, 1937, p. 451) used orcein in alcoholic solution for staining elastin tissue; he employed it for connective tissue, following polychrome methylene blue; and for plasma fibrils in the epithelium, following anilin blue; also with anilin blue or acid fuchsin in studying the process known by him as chromolysis. It has found less frequent use among other histologists; but has been employed in acetic acid solution for staining sections or dissolved in weak hydrochloric acid, for staining sections of embryos; also in combination with various other dyes for bulk staining or as a connective tissue stain. Mollier (1938) calls for it in a quadruple tissue stain, with iron-hematoxylin, naphthol green B and azocarmine G. Kornhauser (1943) uses it in a somewhat different quadruple tissue stain with acid alizarin blue, orange G, and fast green FCF. LaCour (1941) employs it in a stain-fixative for cytological work, either for plants or animals. For this last mentioned purpose the synthetic orcein has not worked as well as that

derived from natural sources; but as an elastin stain, and in the Mollier and Kornhauser quadruple stains, the synthetic orcein seems to be preferable.

For the technic of **the Mollier quadruple stain**, see Staining Procedures, p. IB_3-7.
For the technic of **the Kornhauser quadruple stain**, see Id., p. IB_3-8.
For the technic of **the Tänzer-Unna orcein stain**, see Id. p. IB_3-16.
For **Fraenkel's method for elastic tissue**, see Staining Procedures p, IB_3-17.

U35

LITMUS

The exact composition of litmus is likewise unknown. It is obtained from the same lichens as orcein, treating them with lime and potash or soda, in addition to air and ammonia. Its primary colored principle is known as azolitmin; but there must be other colored compounds in litmus, because the color reactions of azolitmin are not exactly the same as those of litmus. Azolitmin may or may not be a single chemical compound; and in any case, its chemical formula is unknown. It can be synthesized from orcinol by methods very similar to those used in preparing orcein, and the synthetic product is very much like the azolitmin obtained from the lichens, if not identical with it. The color reactions of either litmus or of azolitmin are very similar to those of the indophenols (see p. 102); but it is difficult to figure how an indophenol could be derived from orcinol by the process employed.

Litmus is a feeble dye and is never used as an histological stain. Its classic use is for indicator purposes; at one time it was the best acid-base indicator known. Since the importance of learning actual pH of solutions came to be realized, however, litmus has proved too inaccurate an indicator, and has largely been replaced for chemical use by synthetic dyes (notably the sulfonphthaleins) which change color through a pH-range near the neutral point and which are not subject to loss of color on reduction.

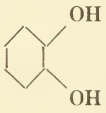
This loss of color by reduction, which is an inconvenience to the chemist who wishes to use litmus as a pH indicator, happens to be of advantage to the bacteriologist. Litmus has been employed for over 50 years as an indicator in bacteriological media; and in some of these media (especially milk) it serves to indicate changes in pH and in O–R potential simultaneously. Although chemically speaking, it is a decidedly inaccurate indicator for either purpose, it has served the bacteriologist to bring out differences between species in a way that no other indicator alone has proved able to do. Azolitmin, either the synthetic product or that derived from the lichens, does not serve the purpose, chiefly because it does not have exactly the same sensitive range in relation to the pH-scale as does litmus.

It is of interest to add that chemists have been telling bacteriologists for years that they should discard litmus in favor of more accurate indicators, using one for pH, another for O–R potential.

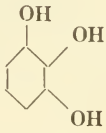
It required the second World War, however, with its interruption of trade and resulting cutting off of the supply of litmus, to stimulate bacteriologists to investigate combinations of indicators to replace litmus. Ulrich (1944) proposed a mixture of methylene blue (to indicate reduction) with chlor phenol red (for pH) which seemed, for some kinds of bacteria at least, to be preferable to litmus as an indicator in milk. This combination, however, shows no sign of replacing litmus for the purpose.

Brazilin and Hematoxylin

The two natural dyes, hematoxylin and brazilin, are closely related chemically and upon decomposition yield the two compounds, pyrocatechol



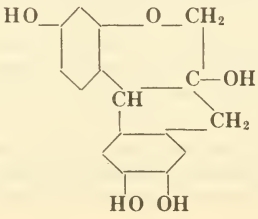
and pyrogallol



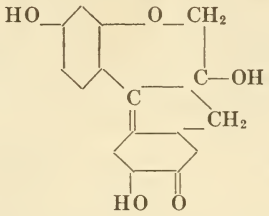
Both dyes are obtained by extraction of the bark of certain trees, hematoxylin from logwood and brazilin from brazil wood (red wood). Both trees are legumes and belong to the family Cesalpiniaceae; they are found only in the tropics. Hematoxylin comes from a single species; while brazil wood is a term applied to various different species all yielding brazilin.

U40 BRAZILIN C. I. NO. 1243

The composition of this substance is supposed to be:



Its solution is colorless, but it becomes red on exposure to the air, as it is then oxidized into the dye brazilein, which probably has the formula:



With alum it is employed as a nuclear stain (known as brazalum) by Mayer (see Lee and Mayer, 1907, p. 218). It is also used by Hickson (1901) for similar purposes following treatment with iron alum as a mordant. Belling (1928) has employed it in place of carmine for staining plant chromosomes in fixed or fresh material, and Shaffer (1933) for distinguishing paper fibers bleached with sulfite from those bleached with sulfate.

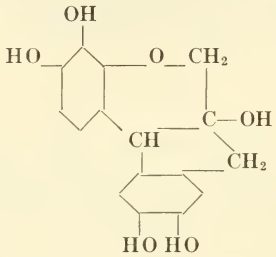
U50 HEMATOXYLIN C. I. NO. 1246

Hematoxylin, as is well known, is a constituent of logwood, a product of South America. It was first obtained about 1840 by extracting logwood chips with ether, evaporating, digesting with alcohol and standing with water after distilling off the alcohol; hematoxylin was found to separate out in crystals. A little later a similar method was employed, but instead of using logwood chips, the starting point of the process was with commercial logwood extract, which is the dried aqueous extract of the wood. In one modern method of manufacture the dried commercial extract is extracted with ether in a continuous extraction apparatus, evaporated to dryness, dissolved in water, filtered and crystallized out of the solution. All of these steps, particularly the ether extraction, are slow and difficult to handle on a factory scale, requiring special expensive apparatus.

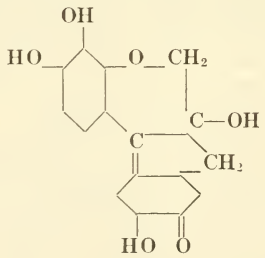
The American manufacture of hematoxylin started during the first World War, just as in the case of the synthetic dyes. The problems presented by its manufacture, however, were quite different from those presented by the synthetics. They were discussed at about that period by McClung (1923) and Conn (1927). Briefly it can be explained here that the first American hematoxylins, marketed during that war, were very crude and proved quite unsatisfactory; but that during the decade following the war, frequent improvement in the supply was made by the manufacturers. The first American product was dark in color; and there was so much demand for a light colored hematoxylin that the manu-

facturers introduced the process of bleaching with sulfur dioxide. It was subsequently found that this bleaching injured the staining qualities, and the manufacturers changed their method and began turning out a darker colored but more satisfactory stain. About 1926 another change in process of manufacture was made and the staining properties seemed thereby to be improved. Until recently there has been only one American manufacturer of hematoxylin whose product could qualify as a biological stain; but the great demand caused by the second World War has resulted in other companies entering the field and developing cheaper processes of manufacture. These cheaper methods of production yield a darker colored and presumably less pure product; it is not, however, to be compared with the crude material put on the American market during the first World War, as it seems to be a satisfactory stain in spite of impurities that may be present.

Hematoxylin is similar to brazilin, but has one more hydroxyl group, the generally accepted formula being:



Like brazilin, it is not a dye, but its color develops in solution upon standing, due to the oxidation into hematein, which is homologous to brazilein and probably has the formula:



Hematein is available commercially in dry form. Less attention has been given to its production than to that of hematoxylin. Hence it frequently results that a hematein sold by some company is more crude than their hematoxylin, although theoretically hematein is a derivative of hematoxylin.

Hematoxylin is without question one of the most important biological stains. It is as valuable to the cytologist and histologist as methylene blue is to the bacteriologist; and probably is second only to methylene blue in the number of different purposes for which it is used. It is valuable not only because it is a powerful nuclear stain and a chromatin stain *par excellence*, but also because it has striking polychrome properties. With the proper differentiation it is possible to get several shades intermediate between blue and red to show in the same preparation.

Hematoxylin is seldom used alone, as it has little affinity for the tissues in itself, even after "ripening" when it is largely converted into hematein. Some form of mordanting is ordinarily required; and most of the hematoxylin formulae either call for some metallic salt or specify previous treatment of the sections with one. In plant histology, however, there is some use for hematoxylin alone. Its greater affinity for plant than for animal tissue implies the presence of aluminium, copper, or iron in the former. In fact hematoxylin can be used as a very delicate reagent for iron or copper.

Perhaps the best known formulae for staining with hematoxylin are the combinations with aluminium, generally in the form of alum. Böhmer's alum hematoxylin (1865), although no longer employed, is of historic interest as it was the first stain of this type to be used. The best known at present is Delafield's alum hematoxylin (see Prudden, 1885), which is a very useful tissue stain with great affinity for chromatin and nuclei, and has much value in staining cellulose walls in vascular plants. Another alum hematoxylin used for similar purposes is that of Ehrlich (see Krause, 1926-7, p. 972).

Mayer's hemalum (1891, 1899) is another well-known alum combination. In this stain hematein is first prepared and then combined with alum. The name hemalum, proposed by Mayer, is now generally accepted for this combination, and various other hemalum formulae have since been proposed. They are useful chromatin stains and are called for in various special procedures.

Mayer (1891, 1899) also combined hematein with aluminium chloride, his hemacalcium calling for this salt and calcium chloride, while his muci-hematein contains aluminium chloride and glycerin. The latter is used for staining mucin.

The iron combinations are perhaps equally valuable. The original iron hematoxylin was that of Benda (1886); but the best known at present is M. Heidenhain's (1892, 1896), which is one of the most useful histological and cytological stains, both in botany and zoology. It is a powerful stain for chromosomes and centrosomes, and is of use for bringing out the middle lamellae in wood. Various other modifications of iron hematoxylin have been used, but they are all similar in principle. Ordinarily the iron salt is

not mixed with the stain, but is used for a preliminary mordanting of the tissue.

Hematoxylin has been combined with chromium, one of the early staining methods being that of R. Heidenhain (1886), which called for potassium bichromate as a mordant. Various recent modifications are in use today, such as that of Apathy (1888), for staining general tissue. Weigert (1884) used a chrom combination for staining nervous tissue.

Benda (1893) employed hematoxylin following treatment with a copper salt for studying spermatogenesis; and Bensley (see Guyer, 1936, p. 164) a similar technic for chromosomes and mitochondria. A formula containing logwood extract, with alum and copper sulfate was suggested by Cook (1879).

Mallory (1938, p. 156) proposed a formula for hematoxylin containing phosphomolybdic acid and also one containing phosphotungstic acid. The latter method is especially valuable for staining cells in the process of mitosis, and for distinguishing fibroglia, myoglia and neuroglia fibrils from collagen and elastin fibrils, especially in tumors, but also in normal tissues. It brings out sharply the striations in skeletal and cardiac muscle fibers. Hematoxylin is used in combination with other stains, especially eosin, but not so frequently as in the case of the common anilin dyes. The Van Gieson technic calls for hematoxylin followed by picric acid and acid fuchsin. A few other methods call for picric acid or ammonium picrate after hematoxylin: and it is sometimes used with eosin or after orange G or acid fuchsin. Most of these combinations, however, are called for only in the case of special procedures.

Isohematein. If hematein is treated with HCl in sealed tubes at 100°C and then treated with silver hydroxide to remove the chlorine, a solution is obtained of a compound known as isohematein, which can be secured as an amorphous mass by evaporation. At the request of the Stain Commission, an American manufacturer of logwood products actually prepared an experimental batch of isohematein by this method, and Cole (1931) reported on its use as a biological stain. The result of this study was to indicate that it could hardly replace hematoxylin as a routine stain, but that on account of its higher tinctorial power it may have value for some special purposes such as the selective staining of nerve cell bodies of fibrillae and cross striations in muscle cells. It has never been made available commercially, and at present there does not seem to be sufficient demand for it to justify its production.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH HEMATOXYLIN AND HEMATEIN ARE USED

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Mayer's mucihematein, Laskey modification	IA_4-8
Delafield's hematoxylin in animal histology	IA_4-11
Heidenhain's hematoxylin in animal histology	IA_4-12
Mayer's hemalum and eosin bluish	IA_4-12
Mayer's hemalum with Congo red	IA_4-13
Mayer's acid hemalum, lillie modification	IA_4-13
Mallory's phosphotungstic acid hematoxylin	IA_4-14
Weigert's iron hematoxylin with safranin	IA_4-6
Hematoxylin with Van Gieson stain	IA_4-21
Crossman modification of Mallory's connective tissue stain	IB_3-5
Hematoxylin with Biebrich scarlet and picro aniline blue	IB_3-12
Mucicarmine with hematoxylin and metanil yellow	IA_4-7
Verhoeff's connective tissue stain	IB_3-14
Modified Gallego elastic tissue stain	IB_3-15
For nuclei in Tänzer-Unna orcein method	IB_3-16
Masson's trichrome stain	IB_3-18
Bielschowsky's connective tissue stain, Foot's modification	IB_3-20
Same; Wilder's modification	IB_3-22
Pal-Weigert method for myelin sheaths	IC_3-28
Hematoxylin with Herxheimer's Sudan IV fat stain	ID_3-18
Lorrain-Smith-Dietrich method for lipoids	ID_3-19
Proescher's oil-red-pyridine for lipoids	ID_3-19
Best's method for glycogen	ID_3-21
Bauer's leucofuchsin method for glycogen	ID_3-22
Heidenhain's hematoxylin for plant histology	IIA-7
Delafield's hematoxylin for plant histology	IA-8
Iron alum hematoxylin and safranin for plant histology	IIA-9
Hematoxylin for permanent smear preparations of sporocytes	IIB-6
Heidenhain's hematoxylin in plant cytology	IIB-8
Regaud's iron alum hematoxylin in plant cytology	IIB-9
Gram-Weigert technic for bacteria in tissues	$IIIB_3$-8
Weigert's stain for fibrin and bacteria in tissues	$IIIB_3$-9
With Verhoeff's carbol fuchsin for tubercle bacilli in tissue	$IIIB_3$-10

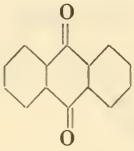
CHAPTER X

MISCELLANEOUS DYES, PIGMENTS, AND HISTOCHEMICAL REAGENTS

1. THE ANTHRAQUINONE GROUP

The anthraquinone dyes include derivatives of anthracene,

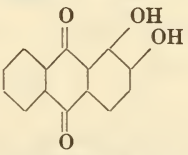
through its oxidation product anthraquinone:



These dyes show the quinoid ring, which is the most important chromophore in nearly all the dyes to be discussed in the three following chapters; it forms very strong chromogens, which require only the addition of auxochrome groups to be converted into strong dyes, either basic or acid. The chromogen anthraquinone is converted into a dye by the addition of hydroxyl groups, its best known derivatives among the dyes being: 1:2 dihydroxy-anthraquinone (alizarin) and 1:2:4 trihydroxy-anthraquinone (purpurin). Both of these compounds occur in nature in the root of madder, being the colored principles of madder extract. They have the property of combining with metallic oxides to form so-called "lakes," insoluble compounds of different color from the dye entering into them. This makes them valuable ones to use after mordanting with aluminium, iron or chromium compounds.

E1 ALIZARIN C. I. NO. 1027

(Various grades denoted as: *Alizarin P, VI, Ie.*)



$C_{14}H_8O_4$; *Mol. Wt.* 240.204

(*An acid dye; absorption maxima about* [*610.8*], *566.5*, [*527.6*] *in alkaline solution*)

Solubility at 26°C: in water nil; in alcohol 0.125%

Alizarin stains tissues a feeble yellowish red if used on them directly. In the presence of aluminium compounds intense red colors are formed; bluish violet in the presence of iron; and brownish violet in the presence of chromium. It has been used as a stain for nervous tissue and for the vital staining of protozoa. Geyer (1932) employs it to distinguish cows' from goats' milk. The chief present use of alizarin, however, is as an indicator.

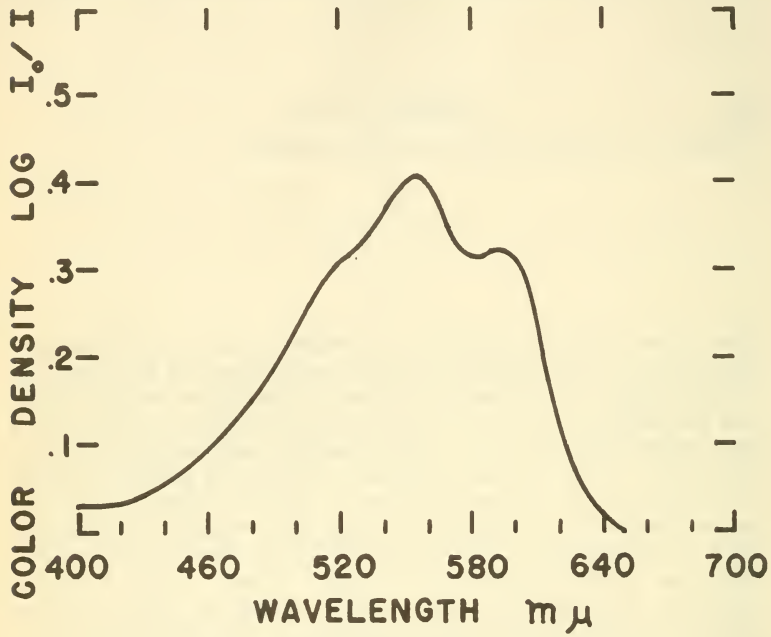
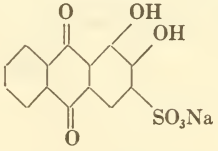


FIG. 26. Spectral curve of alizarin red S.

z5 ALIZARIN RED S C. I. NO. 1034

Synonyms: *Alizarin red, water soluble.* *Alizarin carmine.*



$C_{14}H_7O_7SNa$; *Mol. Wt.* 342.253

(*An acid dye*)

(*Absorption maxima in alkaline solution about 557* [*594–9*])

Solubility at 26°C: in water 7.69%; in alcohol 0.15%

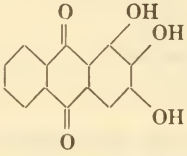
A spectral curve of this dye is given in Fig. 26.

This dye, sodium alizarin sulfonate, was used by Benda for staining chromatin in combination with crystal violet, the chromatin staining brown, while the mitochondria stain violet. It has also been used as a vital stain for nervous tissue in small invertebrates, and by Schrötter (See Lee, p. 465) for sections of nerve tissue. A recent application is for the gross staining of skeletons (Dawson, 1926, Lipman, 1935, Hollister, 1934); in fact, its most frequent modern use is as a stain for bone. Backman (1935) employs it as a chromosome stain in plant cytology.

For various methods calling for this dye in **staining bone**, see Staining Procedures, pp. ID_3-12–17.

E10 PURPURIN C. I. NO. 1037

Synonyms: *Alizarin No.6. Alizarin purpurin.*



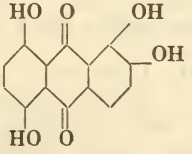
$C_{14}H_8O_5$; *Mol. Wt.* 256.204

(*An acid dye; absorption maxima about* [*521.1*], *485.5*, [*455.5*] *in alcohol.*)

Purpurin is very similar to alizarin, but forms scarlet red lakes with alumina. It has been used as a nuclear stain for histological material, and for determining the presence of insoluble calcium salts in the cell contents.

E11 QUINALIZARIN C. I. NO. 1045

Synonym: *Alizarin Bordeaux BA.*

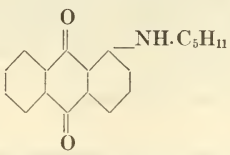


$C_{14}H_8O_6$; *Mol. Wt.* 272.204

(*An acid dye*)

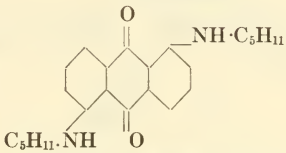
This compound is seldom called for in biological work but has been used by Broda (1939), mixed with azo blue and Titan yellow, as an histochemical reagent for magnesium.

E13 CARYCINEL RED



$C_{19}H_{19}NO_2$; *Mol. Wt.* 293.350

E14 COCCINEL RED

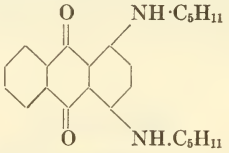


$C_{24}H_{30}N_2O_2$; *Mol. Wt.* 378.496

These are two oil-soluble dyes first described and named by Lillie (1945b). He regarded them, together with the following dye (Oil blue NA) as good fat stains when employed in the supersaturated isopropanol technic (Lillie, 1945 a). Coccinel red stains fat scarlet, carycinel red a deep crimson.

E15 OIL BLUE NA

Probably of the following chemical formula:



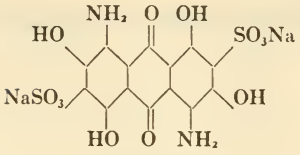
$C_{24}H_{30}N_2O_2$; *Mol. Wt.* 378.496

(*Absorption maxima about* [*595*], *640.*)

This dye has come into prominence since 1940, as a stain for rubber in plant sections. Whittenberger (1944) thus employs it without a counterstain, while Addicott (1944) suggests either safranin or Congo red for counterstaining. According to Lillie (1945 a) it may also be used for staining fat in animal tissue.

For use in **staining fat in tissue**, see Staining Procedures, p. ID_3-20.

E20 ACID ALIZARIN BLUE GR C. I. NO. 1048



$C_{14}H_8N_2O_{12}S_2Na_2$; *Mol. Wt.* 506.334

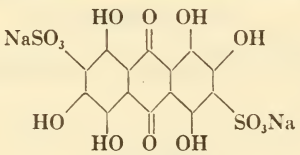
(*An acid dye*)

Some acid alizarin blue, probably either this or the following, has been employed by Buzaglo (1934) in combination with orcein and gallocyanin as a tissue stain. Peterson (1924) has recommended acid alizarin blue in a modification of the Mallory connective tissue stain; the procedure seems to be well worth recommending, but there proves to be some difficulty in standardizing this dye for the purpose.

For the **acid alizarin blue modification of the Mallory technic,** see Staining Procedures, p. IB$_3$-6, 8.

E25 ACID ALIZARIN BLUE BB C. I. NO. 1063

Synonyms: *Anthracene blue SWX, SWX extra*



$C_{14}H_6O_{14}S_2Na_2$; *Mol. Wt.* 508.302

(*An acid dye*)

This dye has come into use in connection with fluorescence microscopy, because of the color which it imparts to various tissue elements under ultra-violet illumination. Some anthracene blue has been called for occasionally in aqueous solution with aluminium sulfate. There are, however, two or three other anthracene blues besides this dye; so it is not certain whether this is the one called for in the technic in question.

E30 ALKANET

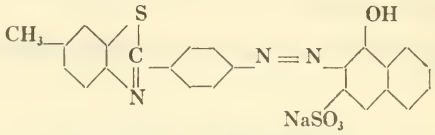
A natural dye of the anthraquinone group, closely related to alizarin. It has rarely been specified for biological use, but Artschwager (1943) has employed it as a stain for rubber in plant sections.

2. THIAZOLE DYES

A small group of dyes of rather complex formula contain the thiazole ring:

in which the indamine group is the chromophore. References to these dyes in biological literature are rare, but the following have been mentioned; two of them, it will be noticed are also azo dyes and thus possess two chromophores.

F5 GERANINE G C. I. NO. 127



$C_{24}H_{16}N_3O_4S_2Na$; *Mol. Wt.* 497.509

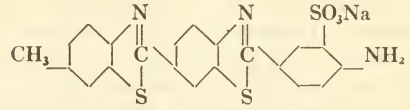
(*An acid dye*)

This is a useful dye in fluorescence microscopy; see Pick (1935) and Jenkins (1937).

F10 PRIMULIN C. I. NO. 812

Synonym: *Primuline yellow.*

A mixture, consisting mainly of the following:



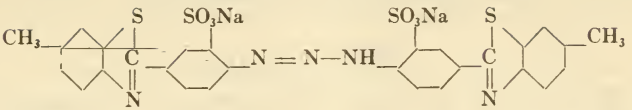
$C_{21}H_{14}N_3O_3S_3Na$; *Mol. Wt.* 475.523

(*An acid dye*)

Among various other thiazole dyes, Pick (1935) employed primulin as a vital stain, for which it is useful because of its fluorescence under ultra-violet illumination. It has since become quite widely used for the purpose.

F15 TITAN YELLOW G C. I. NO. 813

Synonym: *Thiazol yellow.*



$C_{28}H_{19}N_5O_6S_4Na_2$; *Mol. Wt.* 695.706

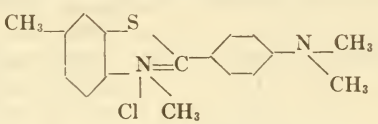
(*An acid dye*)

This dye has been recommended as an indicator for determining magnesium in body fluids. After removing the organic matter and calcium from such solutions the dye is added; under alkaline conditions it becomes red in the presence of magnesium and the color observed can be compared with that of a standard previously prepared.

Also, like the two preceding dyes, it has been employed in fluorescence microscopy; it is one of those recommended by Pick (1935) and by Jenkins (1937) as a vital stain under ultra-violet illumination.

F17 THIOFLAVINE T C. I. NO. 815

Synonym: Thioflavine T G



$C_{17}H_{19}N_2SCl$; *Mol. Wt.* 318.861

(*A basic dye*)

Like the other related dyes here described, this one has properties as a fluorochrome; thus Metcalf and Patton (1944) have employed it, with ultraviolet light, in insect histology.

F20 THIOFLAVINE S C. I. NO. 816

(*An acid dye; a methylated and sulfonated derivative of primulin*)

This is among the dyes that has recently come into use in connection with fluorescence microscopy. Pick (1935) employed a thioflavine as a vital stain, depending on its fluorescence under ultra-violet light; probably he referred to this although there is also a thioflavine T (C. I. No. 815). More recent authors (Jenkins, 1937, Levaditti, et al., 1940) definitely refer to thioflavine S, and

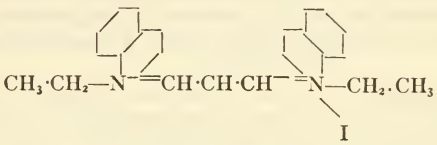
the dye has now come to have a distinct recognition as a fluorochrome. Thus Richards (1943) employs it in the demonstration of the potato scab organism by ultraviolet light.

3. QUINOLINE DYES

Of the quinoline dyes only the following is of interest to biologists:

G5 PINACYANOL C. I. NO. 808

Synonym: *Sensitol red.*



$C_{25}H_{25}N_2I$; *Mol. Wt.* 480.386

(*A basic dye; absorption maxima* [*526, 563*] *608 in alcohol*)

This dye is used primarily for color sensitization in photography, but has been employed by Proescher (1933) for staining frozen sections, and by Hetherington (1936) as a supra-vital stain for mitachondria in blood. It has been employed in combatting streptoccoci. S. H. Bensley (1952) has prepared a neutral stain from it (an erythrosinate) and uses it for staining mast cells.

Another quinoline dye mentioned in biological literature is: Quinoline yellow; C. I. No. 801.

4. DIAZONIUM AND TETRAZONIUM SALTS

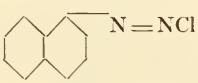
Histochemistry has brought into microscopic use certain compounds which are not dyes. Some of the histochemical reagents, like basic fuchsin, are dyes used in decolorized state, and are discussed elsewhere in this book; others, like the oxidizing agents periodic acid and tetra-acetic acid, are simple chemicals hardly calling for special consideration. There are, however, certain important histochemical reagents which are so closely related to dyestuffs that they can hardly be omitted from these pages. They are especially the diazonium, tetrazonium and tetrazolium salts.

The diazonium and tetrazonium salts have the same basic structure, and are considered in this section. They are salts of a theoretical base having the structure R–N=N–OH [or R–N(OH)≡N]. Not only the base thus indicated, but its simple

salts of mineral acids are very unstable; they can often be stabilized, however, by converting into zinc chloride double salts, and those which are commercially available are ordinarily in this form. In the formulae given below the unstable salt is listed in each case, for the sake of simplicity. The diazonuim salts contain only one $-N=N^+$ group, the tetrazonium salts two. These salts are colorless, but as the azo group ($-N=N-$), is a chromophore, their transformation to colored compounds is easily effected. Their use as histochemical reagents depends on the production of such colored derivatives.

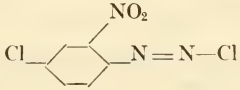
Among the papers introducing diazonium salts into histochemistry is that of Manheimer and Seligman (1948) who proposed a method of demonstrating alkaline phosphatase in normal and neoplastic tissues. This was shortly followed by an adaption of the method to acid phosphatase (Seligman, 1949). The reagent used in this work was:

α-NAPHTHOL DIAZONIUM CHLORIDE



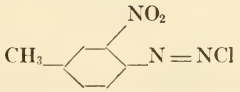
Their histochemical applications are far from standardized, nor is it certain yet which of them will be eventually preferred for the purposes for which they are now employed in a rather experimental way. All that seems advisable to do here is to list, with the formula of the unstabilized salt, those that are known to be at present commercially available.

DIAZO SALT OF P-CHLORO-O-NITROANILINE



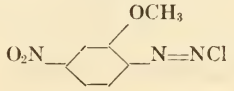
Trade name: *Red 3GS salt* (Nat. Aniline)

DIAZO SALT OF 5-NITRO-2-AMINO NITROBENZENE



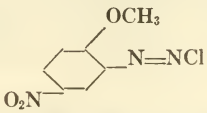
Trade name: *Bordeaux GPS salt* (Nat. Aniline)

DIAZO SALT OF 5-NITRO-2-AMINO METHOXYBENZENE



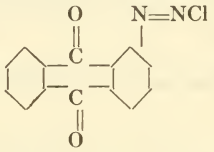
Trade names: *Echtrotsalz B* (Holborn); *Fast red salt B* (Gurr); *Naphthanil diazo red B* (Gurr); *Kernechtrotsalz B* (Gurr); *Nuclear fast red salt B* (Gurr); *Fast red B salt* (Nat. Aniline) See also nuclear fast red salt B in Chap. V (p. 70).

DIAZO SALT OF 4-NITRO-2-AMINO ANISOLE



Trade name: *Scarlet RS salt* (Nat. Aniline)

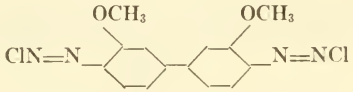
DIAZO SALT OF L-AMINO ANTHRAQUINONE



Trade names: *Naphthanil diazo red AL* (DuPont); *Fast red salt V* (Ciba); *Red ALS salt* (Nat Aniline).

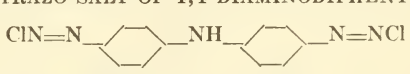
The histochemical use of this reagent for demonstrating esterase, as well as a method for preparing it, as described by Nachlas and Seligman (1949).

TETRAZO SALT OF O-ANISIDINE



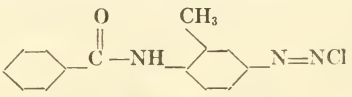
Trade names: *Naphthanil blue salt B, Fast blue salt B, Fast blue salt BN* (Gurr); *Diazo blue B* (DuPont); *Blue BNS salt* (Nat. Aniline).

TETRAZO SALT OF 4,4-DIAMINODIPHENYLAMINE



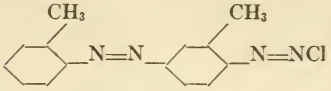
Trade name: *Black BS salt.*

DIAZO SALT OF 6-BENZOYLAMINO-4-METHOXY-3-AMINO TOLUENE



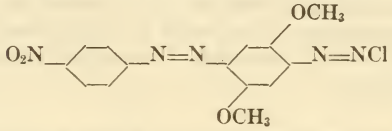
Trade name: *Violet BN salt* (Nat. Aniline).

DIAZO SALT OF AMINOAZOTOLUENE



Trade names: *Fast garnet salt B* (Gurr); *Garnet GBC salt* (Nat. Aniline)

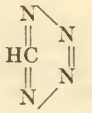
DIAZO SALT OF P-NITROPHENYL-AZO-2,5-DIMETHOXYAMINE



Trade names: *Fast black salt K* (Gurr); *Black K salt* (Nat. Aniline)

5. TETRAZOLIUM SALTS

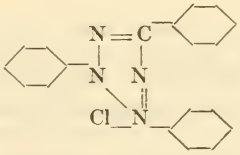
Among the important histochemical reagents, other than dyes, are certain derivatives of tetrazole:



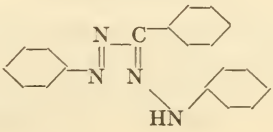
A useful reference to consult concerning the chemistry of these compounds and their biological applications is Smith, F. E. (1951). The earliest prepared and best known of the derivatives is 2,3,5-triphenyl tetrazolium chloride, which is commonly called

TETRAZOLIUM SALT

(*Abbreviation TTC*)



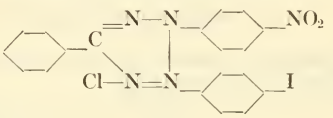
This reduces to the red compound, formazan, a compound containing the azo-group:



The red color produced on reduction was observed, and utilized biologically, before the chemistry involved was understood, because the coloring thereby of certain parts of the embryo in seeds was found to be an indicator of viability of the seeds. It was later learned that other forms of living tissue could bring out the red color; and histochemists discovered that the presence of certain enzymes systems (dehydrogenases) could be visualized by their ability to reduce the tetrazolium salt to formazan. See, for instance, Pratt, Dufrenoy and Pickering (1948).

The compound has, therefore, found considerable histochemical use; but it has certain drawbacks (among them photosensitivity) which have led to investigation of other derivatives of tetrazolium. Among them are:

2-(p-IODOPHENYL)-3-(p-NITROPHENYL)-5-PHENYL TETRAZOLIUM CHLORIDE



This salt is less photosensitive than the preceding, and has the additional advantage (Atkinson, Melvin and Fox, 1950) of slower diffusion into unstained tissue.

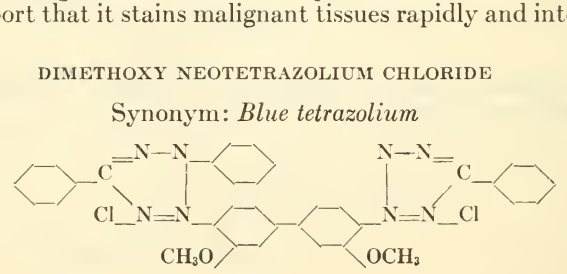
NEOTETRAZOLIUM CHLORIDE

Synonym: *Ditetrazolium chloride*

The reduced form of this salt is deep purple to black, instead of red as in the case of ordinary tetrazolium salt, which makes it easier to distinguish in tissue. Antopol, Glaubach and Goldman (1948) report that it stains malignant tissues rapidly and intensely.

DIMETHOXY NEOTETRAZOLIUM CHLORIDE

Synonym: *Blue tetrazolium*



This like the other tetrazolium salts is colorless itself, but oxidizes into a colored formazan, known in this case as blue formazan, because of its color. This deep blue pigment is a strong indicator of certain types of enzymatic activity, but is much more toxic than ordinary tetrazolium chloride. See Smith (1951).

6. MINERAL PIGMENTS

There are a considerable number of pigments of non-organic nature, which are much better known to the manufacturer of paints than to the biologist. They are in no sense dyes; but a few of them are occasionally used in microscopic work or for some other biological purpose. They were not listed in the first editions of this book because they were regarded as lying outside the field covered.

The following three, however, are occasionally mentioned by the biologist; and they seem worth mentioning, if for no other reason, to keep them from being confused with the dyes. Of these three, the first one is generally known for what it is; but the other two are more apt to be ordered as though they were dyes instead of mineral salts.

PRUSSIAN BLUE C. I. NO. 1288

Synonyms: *Berlin blue. Chinese blue. Paris blue. Milori blue. Steël blue.*

The well-known colored salt, ferric ferrocyanide, $Fe_4(FeC_6N_6)_3$, finds its greatest use in the manufacture of paints, but it has occasional biologic employment, chiefly for coloring anatomical specimens, for demonstration of the circulatory system by means of injection. It is mentioned in connection with the Gömöri (1936) technic in which $K_4Fe(CN)_6$ is used as a microchemical reagent for iron in tissues; by this procedure Prussian blue is formed *in situ* in the tissues.

SKY BLUE C. I. NO. 1286

Synonyms: *Coelestin blue. Coeline. Coeruleum.*

This pigment is essentially cobaltous stannate, $CoOSnO_2$. It is called for primarily as a constituent of artists' oil or water colors, but has been given occasional mention in biological literature.

RUTHENIUM RED

An ammoniated ruthenium oxychloride prepared by making an ammoniacal solution of ruthenic chloride, $RuCl_3$. It has been employed, with or without the addition of acetic acid, in microscopic work, primarily as a test for pectin. It is thought by some to be specific for this purpose.

It should also be mentioned that the name emerald green, listed on p. 142 as a synonym of brilliant green, should more correctly be applied to a hydrated chromium oxide; while Victoria green is a mixture of this compound with two other mineral pigments—although mentioned elsewhere in this book (p. 141) as a synonym of malachite green. These two pigments seem to have no biologic use, but are mentioned here to avoid possible confusion with the two dyes to which the same names have been given.

CHAPTER XI

NEUTRAL STAINS

THERE are two ways in which dyes may be compounded. In the first place it is possible to mix mechanically any two dyes, and if they are of different colors with different selective powers, double staining effects may be procured. In the second place, it is often possible to form a chemical union between two dyes and thus to obtain an entirely new compound which may have quite striking staining properties. It is such compounds as these, rather than simple mechanical mixtures, that are ordinarily referred to as compound dyes.

The simple anilin dyes, it will be recalled (see Chapter II), owe their properties as dyes or as biological stains to the basic or acidic character of the dye molecule. Those parts of the protoplasm which are acid in nature (e. g., chromatin) tend to react with the basic dyes and to be colored by them; while those which are basic (e. g., cytoplasm) react similarly with the acid dyes. (This, to be sure, is not the whole theory of staining, as the process is quite complex and involves physical and mechanical factors as well; but it serves to illustrate the difference between the two kinds of stains.) Now, as already explained, the dyes are not used as free acids or free bases; but rather as sodium or potassium salts of the acid dyes, and as chlorides (or salts of some other colorless acid) of the basic dyes.

It is well known that when two salts, such as sodium chloride and ammonium nitrate, are mixed in solution there is an interchange of ions (metathesis), and the resulting solution, when it reaches equilibrium, contains not only the original salts but also the four free ions and the two alternate compounds as well, in this case sodium nitrate and ammonium chloride. Now if one of these two new compounds happens to be insoluble, as silver chloride for example, which would have been formed if silver nitrate had been substituted for ammonium nitrate, it is thrown out of solution, and equilibrium is not reached until the solution is free (or at least practically free) from the two ions which are insoluble in combination. In the same way, when a sodium salt of an acid dye and a chloride of a basic dye are mixed in solution, there is a similar tendency for the ions to interchange. Ordinarily the dyes are weaker acids and bases than the chlorine and sodium ions respectively; and if the compound dye formed were soluble in water there would be little chance for much of it to be produced. As a matter of fact, however, it is generally insoluble and is therefore thrown out of solution; hence the compound dye can be formed in considerable quantity.

Considering that salts of color acids are called acid dyes, and salts of color bases are known as basic dyes, a theoretically satisfactory name for the compound dyes in which a color acid is combined with a color base would be "neutral dyes". The term is not ordinarily used this way, however; in fact, the dye chemist uses the same term in an entirely different sense. The chemist has not, however, pre-empted the term "neutral stain"; and considering that this class of compound dyes is employed for biological staining rather than general dyeing purposes, the name is quite appropriate. The Stain Commission, therefore, has adopted the use of the term **neutral stain** for this purpose and defines it as a compound of an acid dye and a basic dye in which both the anion and the kation contain chromophore groups.

Ehrlich (1898) introduced a different term, "tri-acid dyes", which he intended, apparently, to apply to the whole class of neutral stains. He seems to have introduced this term on the basis of two assumptions; first, that most of the basic dyes are triamino compounds (and therefore potentially tribasic); second, that in the ordinary mineral acid salts of these dyes only one of the affinities for acid is satisfied, but when compounded with an acid dye the other two affinities also become satisfied. The latter assumption, at least, seems to be entirely incorrect. As just explained, the reaction between an acid dye and a basic dye seems to be purely one of metathesis, and there is no reason whatsoever for assuming that the dye anion attaches itself to any other place in the basic dye molecule than that previously occupied by the displaced mineral anion. Ehrlich's term, however, has persisted for the particular "tri-acid stain" which he specially recommended (see below); and since this particular neutral stain is no more a tri-acid product than any other, some explanation of the term seems necessary.

It is possible to obtain an endless variety of such dyes; but in practice only a certain number of them have proved useful. Among the basic dyes the most suitable for this purpose are the thiazins and the rosanilins (which act as strong ammonium bases); among the acid dyes, the eosins and the sulfonic acids (e.g., orange G and acid fuchsin).

Although the neutral stains are insoluble in water, they are soluble to a greater or less extent in excess of either the acid or the basic dye. Thus if an aqueous solution of acid fuchsin is neutralized by adding drop by drop an aqueous solution of methyl green, there is at first no precipitation, because the methyl green salt of acid fuchsin is kept in solution by the excess of acid fuchsin. After the proper amount of methyl green has been added, however, and the mixture has stood long enough for the reaction to take place, the neutral stain is precipitated and the solution becomes nearly colorless. Then if more methyl green is added the neutral

stain is slowly dissolved again; but as a rule neutral stains are less soluble in excess of basic dye than in excess of acid dye.

As simple aqueous solutions of these compound dyes are impossible and as alcoholic solutions of dyes do not stain well, various methods are employed to secure their action on the tissues. In some instances they are kept dissolved by the presence of an excess of acid or base (particularly the former); in others a certain quantity of acetone or methylal is used to hold the neutral stain in solution; sometimes (as in the original Romanovsky stain) the compound dye is used immediately after mixing, before the reaction is complete or precipitation has taken place; or again (as in the Wright stain) methyl or ethyl alcohol may be used as a solvent, and then after applying the alcoholic solution to the slide it may be diluted with water. This latter method is particularly efficacious, possibly because a temporary state of supersaturation occurs on addition of the water, and there are other known instances where such a state enhances staining power. It should be remarked that when water is mixed with such an alcoholic solution, dissociation must take place and an unstable equilibrium must exist until the compounds insoluble in the mixture of water and methanol have been precipitated; under such circumstances the staining reactions may be rather complicated.

It is assumed that these compound dyes act on the protoplasm somewhat as follows: certain parts of the cell have an affinity for the neutral stain and take it up as such; others, having an affinity for the basic dye, break up the neutral stain so as to obtain the basic portion of it, or if dissociation has taken place, take up the basic ion directly; while other parts of the cell with an affinity for acid dyes similarly combine with the acid portion of the stain. These three types of cell structures are known as neutrophile, basophile and oxyphile elements, respectively. The differentiation thus produced gives the neutral dyes their great value.

Ehrlich's "Tri-acid Stain."

The first neutral stain proposed for microscopic work was the "tri-acid stain" of Ehrlich (1910, II, 313). In forming this compound dye, acid fuchsin and orange G are mixed in solution and to the mixture is then added such a quantity of methyl green that there is still an excess of the acid dye. This excess of the acid dye allows the neutral stain to stay in solution. The dye thus formed is a useful blood stain, and brings out finely the different structures in the leucocytes.

The explanation of the name of this stain (which was based on a mistaken chemical theory) is explained on a preceding page.

Slight modifications of the stain have been used for tissues. The best known is that of Biondi and Heidenhain. (See Krause, 1926-7, p. 457, or Lee, 1937, p. 166).

Thiazin Eosinates*

The first worker to combine eosin and methylene blue was Romanovsky (1891). He realized that a mixture of these two dyes had great selective properties as a stain, and showed it to be excellent for blood, particularly in bringing out the malarial parasite. He also appreciated that it was more than a mere mixture of the two dyes and that some new dye having the property of giving the nuclei a red color was present. It was some time later before the nature of this new dye was known, although it was subsequently named azure I or methylene azure; its true chemistry has scarcely been understood until recently (see p. 107). Methylene violet, and "methylene azure", which probably was also present, had already been described by Bernthsen (1885). How these new dyes were formed in the Romanovsky stain was not known then; although Romanovsky stated that different lots of methylene blue solution varied in their ability to give a good blood stain, and that old solutions on which a scum had formed were best.

One interesting point in connection with this early work is that Bernthsen made the statement that the absorption maximum of Azure I is at 650 mμ. This statement indicates, as can readily be verified by consulting the absorption data of the azures given in Chapter VI, that his azure I may have been primarily azure B. In much recent work concerning the chemistry of azure I, this early statement of Bernthsen's seems to have been overlooked.

Present day blood stains are often spoken of as modified Romanovsky stains; although the modifications are so great as to make them of a very different nature. The first modification was made by Nocht (1898) who concluded that the differential staining was due to the formation of other dyes by the decomposition of methylene blue. Unna (1891) had already described what he called polychrome methylene blue, made by heating a solution of methylene blue on a water bath with potassium carbonate. Nocht decided to use this in the Romanovsky stain instead of untreated methylene blue. He found that it gave very good results if properly neutralized before mixing with eosin; and then learned that better results could be obtained by the use of a smaller amount of alkali and a longer period of polychroming, without subsequent neutralization.

The next step in preparing blood stains was made by Jenner (1899) who collected the precipitate formed when methylene blue and eosin are mixed, and redissolved it in methyl alcohol. He did not use polychrome methylene blue, and his stain lacked the nuclear staining principle of Romanovsky's and Nocht's stains unless allowed to stand for some time before using; but it was an important step in that he showed the possibility of collecting the

*An account of the history of these blood stains is given by MacNeal (1906), and by Conn *et al.* (1933).

precipitated compound stain and of dissolving it in some solvent other than water. Jenner's stain is still called for occasionally. Although it is markedly inferior to the modern type of blood stains when used fresh, it does polychrome on standing in methanolic solution, and this fact undoubtedly explains its continued use.

Combining Jenner's procedure with the Nocht stain was the next logical step and was taken independently by Reuter (1901) and by Leishman (1901). The method thus introduced was briefly to follow Nocht's technic of combining eosin with polychrome methylene blue, but then to filter off the precipitate and to redissolve it in methyl alcohol, not adding further water until the moment of applying the stain to the blood films.

Modern blood stains are in general modifications of Leishman's, differing only in detail. Wright's modification (1902), the one most used in America, (see Mallory and Wright, 1924, p. 170) differs from Leishman's only in that he prepared polychrome methylene blue by heating for only an hour in flowing steam, whereas the Leishman technic calls for twelve hours at 65°C, with subsequent standing for ten days.

Previous editions of this book have contained directions for preparing Wright stain. It is, in fact, perfectly possible for a laboratory worker to prepare a blood stain of this type that gives excellent results; and the technic of doing so is not specially difficult to one who has the necessary experience. Nevertheless, it has proved that the directions given have not yielded uniformly dependable results. It is desirable to have exactly the right amounts of acid and basic dye present to combine without much excess of either; and the relative amounts called for on theoretical grounds do not always yield best results in practice. Moreover, spectrophotometric control during polychroming seems to be necessary to attain a uniform product, and the average laboratory worker does not have the necessary facilities. Furthermore, this stain, either in powdered form or dissolved in methyl alcohol, may be purchased from stain companies at present, usually more uniform in quality than can be made up by the user. Accordingly, directions for its manufacture seem hardly called for here.

Balch's modification calls for a polychrome methylene blue prepared by standing 10 days with precipitated Ag_2O. Hastings modification (1905) differs from that of Wright or Leishman in that the polychrome methylene blue is neutralized with acetic acid, and an unpolychromed solution of methylene blue is mixed with it before eosinating. It is interesting, as a matter of fact, to note that the method of preparing Wright stain followed by some stain manufacturers is more like that of Hastings than like that of Wright. In general American manufacturers sell the same product as "Wright stain" and as "Hastings stain"; perhaps it should all be known by the latter name. Lillie (1944a) has recently suggested the use of a methylene blue polychromed by heating

with a definite proportion of $K_2Cr_2O_7$ in acid solution, a process which he finds to yield a more uniform product.

Giemsa's and MacNeal's modifications are somewhat different. In order to start with a more definite compound than polychrome methylene blue, Giemsa (1902) used more carefully controlled methods of oxidation and obtained a product which he considered to be the same as Bernthsen's Azure I. This he combined with eosin Y, to obtain a more definite compound than in the case of stains of the Leishman type. Subsequently he added methylene blue to the azure before combining with the eosin, and in that way secured better differentiation. He did not state his method of preparing azure I, but apparently gave it secretly to Dr. Grübler's Laboratory, which subsequently became K. Hollborn and Sons; and the latter company made much of this "trade secret" in claiming their Giemsa stain to be the only authentic product of that name. Besides azure I, this company put on the market an azure II which was a mixture of Azure I and methylene blue in equal parts. The eosinate of this mixture, known as azure-II-eosin, was the chief ingredient of Giemsa stain. There probably is no real secret involved in the composition of these azures. Giemsa stated that he used Bernthsen's methylene azure, and Bernthsen described this as having an absorption maximum at 650 mμ; as this is approximately that of present day azure B, we can assume that Giemsa either had the latter or a mixture of higher and lower homologs having optical properties similar to azure B. There has, however, been considerable confusion on this subject of recent years; and in part this has grown out of certain erroneous inferences derived from MacNeal's excellent work (discussed below) on these stains.

The formulæ recommended by Giemsa for preparing solutions of this stain are:

	For blood	For tissue
Azure-II-eosin	3. g.	3. g.
Azure II	0.8 g.	0.8 g.
Glycerol, c.p.	250 g. or 200 ml.	125 g. or 100 ml.
Methyl alcohol, neutral, acetone free	250 g. or 312 ml.	375 g. or 457 ml.

There is apparently a reason for the quantity of glycerin called for in the first of these formulae (50% by weight or about 40% by volume). Recent work in one of the Stain Commission laboratories indicates at least 40% by volume to be necessary to give a reasonably stable solution.

Substituting American dyes for those in the old Giemsa formula, and following the recommendations of Lillie, the following seems advisable:

Azure A eosinate	0.5 g.
Azure B eosinate	2.5 g.
Methylene blue eosinate	2.0 g.
Methylene blue chloride	1.0 g.
Glycerol	375. ml.
Methyl alcohol, reagent	375. ml.

Mixed powdered dyes of approximately this formula are available from some of the American manufacturers.

MacNeal (1922) proposed a stain, intended to give similar results, although prepared on different principles. He showed that by mixing Bernthsen's methylene violet and azure I with methylene blue and eosin Y in definite proportions, dissolving in methyl alcohol, and employing like a Leishman or Wright stain, one could obtain results almost identical with those secured by these other blood stains without the uncertainties introduced by their methods of manufacture. Soon after publishing this paper he realized that methylene azure could be a mixture and certainly was by the methods he had used in preparing it; (MacNeal, 1925). He showed it to contain both azure A and azure B, whose chemistry is discussed in Chapter VI of this book. He regarded the former as the more important ingredient, and stated that the staining effects of Azure B are little different from those of a mixture of azure A and methylene blue. This statement of his gave rise to the impression that azure B was not a satisfactory stain; and only recently has this impression come to be corrected in the minds of users of these stains. MacNeal's tetrachrome stain is ordinarily made up according to the following formula:

Methylene blue chloride (dye content about 90%)	1.0 g.
Azure A	0.6 g.
Methylene violet, Bernthsen, free base	0.2 g.
Eosin Y, (dye content 80–85%)	1.0 g.

It will readily be seen that the dry tetrachrome stain is a mixture rather than a chemical compound like the Leishman stain and its modifications. The four dyes mixed together combine but very slowly even after dissolving in the alcohol; and the first pronounced chemical reaction occurs only after diluting with water in actual application. There is evidence, however, that certain chemical reactions (and of an undesirable nature) take place in the alcoholic solution, and thus interfere with the keeping qualities of the solution. Manufacturers of tetrachrome stain realize this and give directions for allowing the alcoholic solution to stand 48 hours at 50°C and then filtering; this practice seems to remove whatever harmful compound may have formed, and the solution thus obtained is reasonably permanent. Undoubtedly, moreover, methyl alcohol alone is an undesirable solvent for this mixture of dyes, just as it is in the case of Giemsa stain. There can be little question but that the addition of 40% glycerol, by volume, as recommended by Giemsa, would make the solvent for the tetrachrome stain more satisfactory and would improve the keeping qualities of the solution.

It can readily be inferred that the thiazin eosinates, made up by any of the above mentioned procedures, must be very complex mixtures. Theoretically each of the azures or other oxidation

products of methylene blue present in the polychromed solution may combine individually with the eosin, thus yielding several different eosinates in the precipitate. Unless the precipitate is washed with two or three changes of water it usually contains a considerable excess of one or the other of the uncombined dyes, and this may further increase the complexity of the mixture. If properly washed, the precipitate should be free, or almost free, of uncombined basic or acid dye, because of their great solubility, and possibly such is also the case in the methanolic solution of the precipitate; but it is certain that when water is added in the process of staining, dissociation must occur with the formation of the acid and basic dyes from which the compound was derived. This is another contribution to the complexity of the solution with which the staining is accomplished. Complex as the mixture is, Lillie (1942) has shown that it can be quite effectively analyzed spectrophotometrically; and that from the position and width of the absorption bands, one can get some idea as to the composition of the polychrome methylene blue from which the eosinate was prepared. By comparing such spectrophotometric results with the behavior in staining, one can obtain practical information as to the value of the different azures in a stain of this type.

In addition to the chemistry of the constituent dyes, another important factor in a blood stain of this type is the nature of the solvent. Methyl alcohol, as explained above, is the usual solvent employed; but there are many grades of this reagent available, and not all are equally suitable for the purpose—a fact which at one time was not fully appreciated. Apparently absolute purity of the methanol is not needed; but one point is very important—the solvent must be neutral in reaction. Methanol, as formerly prepared by the destructive distillation of wood, often contained considerable amounts of the other two major products of that decomposition, acetone and acetic acid. The presence of acetone seems to be unimportant (in spite of previous recommendations that methanol for blood stains should be acetone-free); acetic acid, however, is quite deleterious in that it tends to precipitate eosin as its color acid, when the methanolic solution is diluted with water. Special methyl alcohol for blood stains is on the market, but the modern synthetic methanol of reagent grade proves quite satisfactory; its further purification with Ag_2O, as sometimes recommended to free it from aldehydes and ketones, seems to be quite unnecessary. (See Lillie, 1944b).

The two blood stains for which there is now most demand in America are Wright stain and Giemsa stain, while the tetrachrome stain is used less frequently. Wright stain and the tetrachrome stain both give almost identical results and are handled very similarly. If one is to prepare the stain himself, there are reasons for preferring the tetrachrome stain as it can be

made up with more certainty of obtaining a satisfactory product. Ordinarily today, however, one purchases his blood stains already prepared by some manufacturer; and the Wright stain on the market seems to be as reliable as the tetrachrome stain.

Giemsa stain, although originally intended to be more definite in composition than other blood stains, is possibly the most complicated of them all. As a simple stain for blood in thin smears, some find it to have no distinct advantage over stains of the Leishman-Wright type, although others claim it to show greater purity of color and sharpness of definition, with deeper staining of chromatin; such advantages as it has for this purpose are offset by the longer staining time and larger volume of solutions required. As a stain for malarial blood, however, designed to show the malaria plasmodium, Giemsa stain is definitely preferred. In searching for the malaria organism the present approved technic calls for the use of thick smears of blood in which laking of the red corpuscles is brought about by distilled water (either as a diluent of the stain or placed on the smears before staining); accordingly a stain for the red cells is not required, but one is necessary that brings out the leucocytes and the plasmodia. For this, a properly prepared Giemsa stain has decided advantages over Wright stain. Good results have quite consistently been obtained with the German Giemsa stain, when it has not become unavailable because of war; and there is good reason to believe that it has been quite constant in composition. As remarked above, its manufacturers used to lay considerable stress on the "secret" character of azure I and to claim that since they alone know that secret, no other Giemsa stain except theirs could be reliable. As mentioned above, however, no real secret seems to have been involved; and recent investigations have shown that a Giemsa stain, having one of its absorption maxima at about 655, as recommended by Lillie (whether prepared in the laboratory or put on the market as a commercial product) is an excellent malarial stain, as judged by technicians familiar with the technic in question.

During the two decades from 1920 to 1940, however, much Giemsa stain was on the market in America which did not conform to the requirements now specified by Lillie. This was largely due to the stress laid by MacNeal on Azure A as the important ingredient of azure I, and to the fact that Holmes and French later (1926) said quite definitely that azure B has no staining value. It took some time to counteract the effect of such statements, and meanwhile American manufacturers of Giemsa stain, with the endorsement of the Stain Commission, used azure A, without any appreciable azure B, in its preparation. Since Lillie has called attention to the importance of azure B, however, the practice of the stain companies has changed; and now there is an American

Giemsa stain available which gives results very much like the imported product.

Other uses of thiosin eosinates. Although thiazin eosinates are specially known for their use as blood stains, they are coming more and more to be employed for other purposes. Tolstoouhov (1928), for instance, recommended a Romanovsky mixture as a tissue stain, his solution proving stable because of the excess of methylene blue present; he showed that such a mixture can produce a variety of staining effects according to the reaction given to the solution by adding varying quantities of N/100 NaOH or HCl. The same author (1929) later proposed a similar mixture for differentiating one kind of bacteria from another. Churchman (1933) has employed Wright stain for demonstrating bacterial capsules, and Dutton (1928) to stain bacterial spores. Giemsa stain has been used similarly, and also as a tissue stain. Lillie and Pasternack (1932, 1936) have, in fact, shown that various thiazin eosinates can be used very effectively as a tissue stain; Lillie (1941) has given in detail a technic for staining tissue with a specially modified Wright stain following hematoxylin. His latest recommendations (1944c) call for azure A, azure C or toluidine blue salts of eosin Y or eosin B, with special preference for those of eosin B which are stated to give deeper red staining of hemoglobin and granules.

PROCEDURES RECOMMENDED BY THE COMMISSION IN WHICH THIAZIN EOSINATES ARE USED*

NAME OF PROCEDURE	PAGE REFERENCE TO STAINING PROCEDURES
Buffered azure eosinates for tissue	IA_4-17
Wright stain for blood smears	ID_3-3
MacNeal's tetrachrome stain for blood smears	ID_3-5
Giemsa stain for thin films	ID_3-6
Giemsa stain for thick films	ID_3-6
Giemsa or Wright stain for bone marrow smears	ID_3-10, 11

*Under this heading are given references to procedures described in detail in Staining Procedures, edited by Conn and Darrow (1943-4).

Other Compound Stains

Various other compounds of acid and basic dyes have been used for special purposes. The basic dyes employed in these compounds most commonly are perhaps methyl green and methylene blue; but sometimes basic fuchsin, pyronin or rhodamine or even a weak base like neutral red is used. Most common among the acid dyes in these compounds are eosin, orange G and acid fuchsin; but certain others are occasionally employed. Picric acid forms a few useful compound dyes, rosanilin picrate (i. e. the compound

of basic fuchsin and picric acid) being especially well known as a tissue stain.

The Pappenheim panoptic triacid stain is a modification of Ehrlich's triacid compound. In this combination methylene blue or methylene azure is substituted for methyl green. It is a tissue stain of use in certain special technics. Another well-known neutral stain is the Twort (1924a and b) formula in which neutral red and light green are combined. The Twort stain is valuable for staining animal parasites and other animal organisms in tissues. Maurer and Lewis (1922) (see page 165) have combined safranin in a neutral stain with some acid violet and have employed the compound dye in staining glandular tissue.

Ehrlich proposed various other neutral stains, the best known being a compound of acid fuchsin and methylene blue used for staining blood; and a compound of narcein, an acid dye, with two basic dyes pyronin and methyl green or methylene blue. A combination of orange G with a gentian violet has been proposed by Bensley (1911) as a stain for the A and B cells in the islands of Langerhans.

APPENDIX I

TABLES RELATING TO STAINS

How to Use These Tables

Identifying a dye from its index number. Dyes in England and the United States are generally designated by their Colour Index number, in Germany and several other countries by their Schultz number. If the C. I. number alone is known for one of the biological stains, it can be identified by Table 1, in which the dyes are arranged in the order of the C. I. numbers; if known by the Schultz number it can be identified by Table 2, in which the Schultz numbers of these same stains are arranged serially, followed by the corresponding C. I. numbers and page references to where the dyes are described in this book.

Table 1 can also be used in studying the *synonymy* of the stains; but will not prove as convenient as the general index for identifying a dye from some synonym, as the arrangement is not alphabetical.

Learning the common uses of stains. In Table 3 the dyes are listed under their preferred designations in the order in which they occur in the main part of this book. In the second column are given their principle uses as stains. References to the authors of the procedures are not given in this table, but can be obtained by consulting the page in the main part of the book where the dye in question is described.

Selecting stains for some general field of work. Table 5 includes lists of the dyes most commonly used in general fields of microscopic work, e.g. animal histology, plant histology, etc. It should prove useful in selecting the stains needed in equipping a new laboratory in some one of these fields.

Learning the solubility of a stain. In making solutions of stains it is often desirable to know their solubility in either water or 95% alcohol. Figures indicating these solubilities of recrystallized dyes are given in Table 6, which includes all the most commonly used stains, and numerous others; the arrangement in this table is alphabetical. The solubilities of commercial dyes, however, vary widely from these figures for the purified products; the former are ordinarily less soluble, though occasionally the opposite. A limited amount of information is available, thanks to the coöperation of the American Pharmaceutical Association, as to the solubilities of commercial samples of about 25 of the commonly used stains; this is given in Table 7, and in some cases may prove more useful in making up a staining fluid than that given in Table 6.

TABLE 1. NOMENCLATURE AND SYNONYMY OF STAINS.
Arranged in order of their Colour Index Numbers

Colour Index No.	Schultz* No.	Preferred Designation	Synonyms; and slightly varying shades	Page reference
		(a) Synthetic Dyes		
2	2	Naphthol green Y	Fast printing green. Gambine.	65
5	5	Naphthol green B	Naphthol green. Green PL. Acid green O.	66
7	9	Picric acid		66
9	18	Martius yellow	Naphthol yellow. Manchester yellow.	68
12	15	Aurantia	Imperial yellow.	68
..	..	Nuclear fast red salt R		70
16	172	Fast yellow	Acid yellow. Fast yellow FY, G, S, BG, etc.	70
19	28	Oil yellow II	Butter yellow. Oil yellow D. Fast oil yellow B.	71
20	27	Chrysoidin Y	Brown salt R. Dark brown salt R.	71
..	..	Benzene-azo- *a*-naphthylamine		72
27	39	**Orange G†**	Wool orange 2G, Crystal orange GG. (Slightly differing grade: Orange GG, GMP).	72
29	44	Chromotrope 2R	Chromotrope N2R. Chromotrope blue 2R. XL Carmoisine 6R. Fast fuchsin G. Acid phloxine GR.	73
31	..	Azophloxine GA	Fast crimson GR. Amido-naphthol red G.	73
54	105	Sorbine red	Azofuchsin 3B. Kiton red S. Eriorubine G. Azo acid red L. Azo rhodine 3G.	74
59	104	Azo acid blue B		75
..	..	Nitrazine	Nitrazine yellow. Delta dye indicator.	76
73	92	Sudan II	Oil scarlet. Fast oil orange II. Red B. Fat ponceau. Orange RR.	76
79	95	Ponceau 2R	Ponceau R, RG, G, 4R, 2RE, NR, J, FR, GR. Scarlet R. Xylidine ponceau 3RS. Lake ponceau. Brilliant ponceau G. New ponceau 4R.	77
81	115	Oil brown D	Sudan brown. Sudan brown AN. Fast oil brown S. Brilliant fat brown B. Fat brown III.	77
88	123	Bordeaux red	Fast red B, BN or P. Cerasin R. Archelline 2B. Azo-Bordeaux. Acid Bordeaux.	78
113	149	Sudan R	Brilliant fat scarlet B. Oil vermillion.	78
127	264	Gerinine G		230
133	282	Janus green B	Diazin green S. Union green B.	78

134	283	Janus black	Diazine black.	80
138	169	Metanil yellow	Orange MNO or MN. Acid yellow R. Soluble yellow OL. Yellow M. Tropaeolin G.	80
142	176	Methyl orange	Orange III. Helianthin. Gold orange MP. Tropaeolin D.	81
..	..	Prontosil		81
143	179	Orange IV	Orange N. Acid yellow D. Tropaeolin OO.	82
144	181	Brilliant yellow S	Curcumine. Yellow WR.	82
150	185	Orange I	Naphthol orange. Tropaeolin G, OOO No. 1.	82
151	189	Orange II	Gold orange. Orange A, P, R. Acid orange II, Y or A. Orange extra. Mandarin G. Tropaeolin OOO No. 2.	83
152	187	Narcein		83
184	212	Amaranth	Naphthol red S, C or O. Fast red. Bordeaux. Bordeaux SF. Victoria rubin O. Azo rubin. Wool red.	84
211	250	Methyl red		84
225	277	Thiazine red R	Chlorazol pink Y. Rosophenine 10B.	85
248	532	**Sudan III**	Sudan G. Tony red. Scarlet B, fat soluble. Fat ponceau G. Oil red AS, O, B or 3B. Cerasin red.	86
252		Brilliant croceine	Croceine scarlet 3B, MOO.	86
258	541	**Sudan IV**	Scarlet red. Fat ponceau. Fat ponceau R or LB. Cerotine ponceau 3B. Oil red IV.	87
..	..	Oil red O		88
..	..	Oil red 4B		88
264		Naphthol blue black	Pontacyl blue-black SX, Buffalo black NBR.	85
280	569	Biebrich scarlet, water soluble	Croceine scarlet 5R. Ponceau B. Double scarlet BSF. Scarlet B, or EC.	89
282		Ponceau S.	Fast ponceau 2B.	89
284	572	Orseillin BB		90
331	311	Bismarck brown Y	Vesuvin. Phenylene brown. Manchester brown. Excelsior brown. Leather brown. Basic brown G, GX or GXP. (Slightly different shade: Bismarck brown G).	90
332	318	Bismarck brown R	Bismarck brown GOOO. Brown R, AT, C or N. Manchester brown EE. Vesuvin NR, B, R. Basic brown BR or BXN.	92
370	360	Congo red	Congo. Cotton red B or C. Direct red C, R or Y.	92

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†The dyes printed in bold face type are the most commonly used stains.

TABLE 1. (*Continued*)

Colour Index No.	Schultz* No.	Preferred Designation	Synonyms; and slightly varying shades	Page reference
375	365	Erie garnet B	Congo corinth G or GW. Corinth brown G. Cotton corinth G. Amanil garnet H. Direct garnet R. Buffalo garnet R. Direct violet C. Diamine Bordeaux CGN.	93
438	434	Trypan red		93
448	448	Benzopurpurin 4B	Cotton red 4B. Dianil red 4B. Diamin red 4B. Sultan 4B. Direct red 4B.	93
454	449	Brilliant purpurin R		94
456	452	Vital red	Brilliant Congo R. Brilliant Congo red R. Brilliant vital red. Acid Congo R. Azidine scarlet R.	94
463	460	Azo blue	Benzoin blue R. Direct violet B.	95
465	476	Dianil blue 2R	Direct steel blue BB. Benzo new blue 2B. Naphthamine brilliant blue 2R.	95
477	471	Trypan blue	Chlorazol blue 3B. Benzo blue 3B. Dianil blue H3G. Congo blue 3B. Naphthamine blue 3BX. Benzamin blue 3B. Azidin blue 3B. Niagara blue 3B.	96
..	..	Evans blue		96
..	..	Vital new red		96
520	513	Niagara blue 4B	Pontamine sky blue 5BX. Direct sky blue. Benzo sky blue.	97
..	..	Marshall red		97
..	..	Hickson purple		97
581	671	Chlorazol black E	Pontamine black E, EX or EXX. Erie black GXOO, B, or BF. Direct black MS, RL, E, GX. Direct deep black EW extra, E, EA, EAD extra. Renol black G.	97
..	..	Sudan black B		98
..	..	Victoria green G		99
655	752	Auramine O	Pyoktaninum aureum. Pyoktanin yellow. Canary yellow.	139
657	754	Malachite green	Victoria green. New Victoria green extra, O, I or II. Diamond green B, BX, or P extra. Solid green O. Light green N.	141
662	760	Brilliant green	Ethyl green. Malachite green G. Emerald green crystals. Solid green JJO. Diamond green G.	142
670	765	Light green SF, yellowish	Light green 2 G, S, 2GN. Acid green (with various shade designations). Fast acid green N.	143

...	...	Fast green FCF			144
676	779	**Pararosanilin†**	**Basic fuchsin‡**	Basic rubin. Parafuchsin. Paramagenta. Anilin red. Magenta O.	147
...	...	Rosanilin		Magenta I.	
677	780	Mixture of rosanilin and pararosanilin		Magenta.	147
...	...	Magenta II			
678	782	New fuchsin		Isorubin. Fuchsin NB. Magenta III.	148
679	784	Hofmann's violet		Dahlia. Iodine violet. Primula R. Red violet. Violet R, RR, 4RN.	155
680	783	**Methyl violet**		Dahlia B. Paris violet. Pyoktaninum coeraleum. (Various shades denoted: Methyl violet 2R, R ,B, 2B, 3B, BBN, BO, and V3).	156
681	785	**Crystal violet**	**Gentian violet¶**	Violet C, G or 7B. Hexamethyl violet. Methyl violet 10B. Gentian violet.	160
682	787	Ethyl violet		Ethyl purple 6B.	161
684	788	**Methyl green**		Double green SF. Light green.	162
685	789	Ethyl green		Methyl green.	164
686	790	Iodine green			164
689	792	Spirit blue		Anilin blue, alcohol soluble. Gentiana blue 6B. Light blue. Lyon blue. Paris blue.	166
690	793	Victoria blue 4R		Fat blue 4R.	167
692	800	**Fuchsin, acid**		Fuchsin S, SN, SS, ST, or S III. Acid magenta. Acid rubin.	153
706	815	Methyl blue		Cotton blue. Helvetia blue.	167
707	816	**Anilin blue, water soluble**		China blue. Soluble blue 3M or 2R. Marine blue V. Cotton blue. Water blue.	168
710	817	Isamine blue			170
712	826	Patent blue V		Alphazurine 2G. Patent blue VF. Pontacyl brilliant blue V.	170
715	828	Xylene Cyanol FF		Cyanol FF	171

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‡At least three different dyes are apparently sold to biologists as basic fuchsin.

¶Nearly synonymous with methyl violet; various mixtures of methyl and crystal violet are sold under this name.

TABLE 1. (*Continued*)

Colour Index No.	Schultz* No.	Preferred Designation	Synonyms; and slightly varying shades	Page reference
724	843	Rosolic acid	Aurin. (Yellow corallin is its sodium salt.)	172
726	844	Red corallin	Aurin R.	173
727	846	Chrome violet CG		173
728	821	Victoria blue R	New Victoria blue B or R. Corn blue B.	173
729	822	Victoria blue B	Fat blue B. Corn blue BN.	174
731		Nigbt blue		174
737	836	Wool green S	Wool green BS, BSNA or C. Lissamine green B, BS. Pontacyl green S. Acid green S. Cyanol green B. Fast light green. Calcoid green S extra.	175
739	853	Pyronin Y	Pyronin G.	178
740	854	Acridine red 3B		178
	855	Pyronin B		180
	864	Rhodamine B	Rhodamine O. Brilliant pink.	181
752	866	Rhodamine 6G	Rhodamine 6GX and 6GDN extra. Calcozine red 6G extra.	182
758		Violamine R	Acid violet 4R. Fast acid violet 3RL, A2R and R.	182
760	872	Fast acid blue R	(Almost the same as Violamine 3B).	183
766	880	Fluorescein	(Uranin is its sodium salt.)	186
768	881	**Eosin, yellowish**	Eosin, water soluble. Bromo acid, J, TS, XL or XX. Bromo fluorescein. Bronze bromo ES. Various shades denoted: Eosin B extra, BP, BS, DH, G, GGF, J extra, 3J, 4J, JJF, KS, S extra, Y extra and YS.	187
769	882	Methyl eosin	Eosin, alcohol soluble.	189
770	883	Ethyl eosin	Eosin, alcohol soluble. Eosin S.	190
771	885	Eosin B (i.e., bluish)	Eosin BN, BA, BS, BW, DHV. Saffrosin. Eosin scarlet. Scarlet J, JJ, V. Nopalin G. Imperial red. Eosin scarlet B.	191
...	...	Mercurochrome 220		192
772	886	Erythrosin, yellowish	Erythrosin R or G. Pyrosin J. Iodeosin G. Dianthine G.	192
773	887	Erythrosin, bluish	Erythrosin B, N or JN. Pyrosin B. Eosin J. Iodeosin. Dianthine B. (Slightly different shades: Erythrosin D, J, JNV, W).	193
774	888	Phloxine	Erythrosin BB, or B extra. New pink.	195
777	889	Rose bengal G	Rose SA. (Various shades denoted: Rose bengal N, AT, NT, NTO, and B).	196
778	890	Phloxine B	Phloxine TA, N, BP super, RB, TB, or BB. Cyanosine. Eosin 10B.	195

779	891	Rose bengal	(Various shades denoted as: Rose bengal extra, B, 2B, 3B conc., DY, N extra.)	197
785	901	Acridine yellow		206
787	903	Coriphosphine O		207
788	902	Acridine orange NO	Basic orange 3RN. Euchrysine 3RXA.	207
	906	Rivanol		207
790	906	Acriflavine	Trypaflavine. Flavine.	208
...	...	Neutral acriflavine	Neutral trypaflavine. Euflavine. Neutroflavine. Gonocerin.	208
790	906	Proflavine		208
...	...	Atabrine		209
793	910	Phosphine	Leather yellow. Xanthene.	209
795	911	Rheonine A	Rheonine AL, G or N. Fast phosphine NAL.	210
808	924	Pinacyanol	Sensitol red.	232
812	932	Primulin	Primulin yellow.	230
813	280	Titan yellow G	Thiazol yellow.	231
815		Thioflavine T	Thioflavine TG	231
816	933	Thioflavine S		231
819	938	Bindschedler's green		102
820	937	Toluylene blue		102
821	939	Indophenol blue	Indophenol	103
825	946	Neutral red	Toluylene red.	126
826	945	Neutral violet		128
828	949	Azocarmine G	Azocarmine GX. Rosazine. Rosinduline GXF.	132
840	958	Phenosafranin	Safranin B extra.	128
841	967	**Safranin O**	Gossypimine. Cotton red. Safranin Y or A. (Slightly different shades: Safranin AG, T, MP, and G.)	129
847	960	Amethyst violet	Heliotrope B. Iris violet.	131
857	976	Magdala red	Naphthalene red. Naphthalene pink. Naphthylamine pink. Sudan red.	133
860	982	Indulin, spirit soluble	Indulin (with various shade designations). Spirit indulin. Spirit nigrosin R.	134
861	984	Indulin, water soluble	Indulin (with various shade designations). Soluble indulin 3B. Fast blue B, OB, R, 2R, etc.	134

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TABLE 1. (*Concluded*)

Colour Index No.	Schultz* No.	Preferred Designation	Synonyms; and slightly varying shades	Page reference
865	986	Nigrosin, water soluble	Nigrosin W, WL, etc. Gray R, B, BB. Silver gray. Steel gray. Indulin black.	134
877	992	Brilliant cresyl blue	Cresyl blue 2RN or BBS. Brilliant blue C.	117
883	998	Gallocyanin	Alizarin blue RBN. Chrome blue GCB. Fast violet.	119
894	1009	Gallamin blue		119
900	1015	Celestin blue B	Coreine 2R.	120
908	1022	Resorcin blue	Fluorescent blue. Iris blue. Often called lacmoid.	120
913	1029	Nile blue sulfate	Nile blue A	121
...	....	Cresyl violet	Cresyl echt violet.	122
909	1025	New blue R	Naphthol blue R. Fast blue 3R. Phenylene blue. Meldola's blue. Indin blue 2RD.	124
...	....	Resazurin		124
920	1036	Thionin	Lauth's violet. (Not thionin blue, which is Schultz No. 661.)	104
922	1038	**Methylene blue**†	Swiss blue. (Slightly different grades: Methylene blue BX, B, BG and BB. Grade preferred for staining: Methylene blue chloride)	111
		Azure C		107
923	1039	Azure A	Methylene azure. Azure I.	109
		Azure B		110
...	1038 (note)	Methylene violet, Bernthsen	(Not methylene violet RRA or 3RA, Schultz No. 680.)	114
924	1040	Methylene green		115
925	1041	Toluidine blue O	Methylene blue T50 or T extra.	115
927	1043	New methylene blue N	Methylene blue NN.	116

1027	1141	Alizarin	(Various grades denoted as: Alizarin P, VI, Ie).	225
1034	1145	Alizarin red S	Sodium alizarin monosulfonate. Alizarin red water souble. Alizarin carmine.	226
1037	1157	Purpurin	Alizarin No. 6. Alizarin purpurin.	227
1045	1168	Quinalizarin	Alizarin Bordeaux BA.	227
....	....	Oil blue NA		228
1240 (note)	1382	Alkanet		229
1048	1170	Acid alizarin blue GR		229
1063	1175	Acid alizarin blue BB		229
		(b) Natural Dyes		
....	....	Saffron		211
1177	1301	Indigo	Indigo blue.	211
1180	1309	Indigo carmine	Indigotine Ia.	212
1237	1373	Berberine		212
1239	1381	**Cochineal**	(The aqueous extract of the cochineal insect.)	213
		Carmine	(The lake prepared by adding alum to cochineal.)	214
		Carminic acid	(The active dye purified from cochineal.)	214
1242	1386	Orcein		216
		Litmus		217
1243	1375	Brazilin		218
1246	1376	**Hematoxylin**	(The ether extract of logwood.)	219
		Hematein	(The dye formed on oxidation of hematoxylin.)	237
1286	1443	Sky blue .	Coelestin blue. Coeline. Coeruleum. ($CoOSnO_2$).	237
		Ruthenium red	($RuCl_3$).	
1288	1436	Prussian blue	Berlin blue. Chinese blue. Milori blue. Steel blue. $[Fe_4(FeC_6N_6)_3]$.	237

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Table 2. Numbers Used in Schultz' Farbstofftabellen, 7 Ed.

Compared with Colour Index numbers of dyes described in this book

Schultz 7 Ed.	Colour Index	Schultz 7 Ed.	Colour Index	Schultz 7 Ed.	Colour Index
2	2	758	729	906	790
5	5	760	662	910	793
8	7	765	670	911	795
9	7	779	676	924	808
14	12	780	677	932	812
15	12	781	676	933	816
18	9	782	678	937	820
21	9	783	680	938	819
27	20	784	679	939	821
28	19	785	681	945	826
39	27	787	682	946	825
44	29	788	684	949	828
92	73	789	685	958	840
95	79	790	686	960	847
104	59	792	689	967	841
105	54	793	690	976	857
115	81	798	676	982	860
123	88	800	692	984	861
149	113	815	706	986	865
169	138	816	707	992	877
172	16	817	710	998	883
176	142	821	728	1009	894
179	143	822	729	1015	900
181	144	826	712	1022	908
185	150	828	715	1024	908
187	152	836	737	1025	909

189	151	843	724	1029	913
212	184	844	726	1036	920
250	211	845	677	1038	922
264	127	846	727	1039	923
277	225	853	739	1040	924
280	813	854	740	1041	925
282	133	855	741	1043	927
283	134	856	739	1141	1027
311	331	864	749	1145	1034
318	332	866	752	1157	1037
360	370	872	760	1158	1037
365	375	880	766	1160	1037
434	438	881	768	1168	1045
448	448	882	769	1170	1048
449	454	883	770	1175	1063
452	456	885	771	1301	1177
460	463	886	772	1306	1177
471	477	887	773	1308	1177
476	465	888	774	1309	1180
513	520	889	777	1373	1237
532	248	890	778	1375	1243
541	258	891	779	1376	1246
569	280	894	766	1380	1239
572	284	896	768	1381	1239
671	581	901	785	1386	1242
752	655	902	788	1436	1288
754	657	903	787	1443	1286

TABLE 3. THE PRINCIPAL USES OF THE MOST IMPORTANT STAINS

Name of stain and page reference*	Application
Naphthol green Y p. 65 or Naphthol green B p. 66	With gentian violet and azocarmine G; as tissue stain.
	With orcein, iron-hematoxylin and azocarmine G; as tissue stain.
Picric acid p. 66	For cytoplasm. Contrast stain. Often in solution with anilin blue W.S.
	With acid fuchsin; Van Gieson's connective tissue stain.
	With crystal violet; in cytology of fungi.
	Staining cell walls of marine fungi.
Martius yellow p. 68	Pianese's stain for cancer tissue; with acid fuchsin and malachite green.
	Same technic adapted for staining pathologic plant tissue (fungus diseases).
	In contrast to resorcin blue for staining pollen tubes in styles.
Aurantia p. 68	For light filters in photomicrography.
	Champy-Kull technic for mitochondria; with acid fuchsin and toluidine blue.
	In studying polyploidy in plants.
Fast yellow p. 70	For bone sections.
	Combined with eosin, phloxine and anilin blue; in the study of chromolysis by Unna.
	With hematoxylin and acid fuchsin; as tissue stain.
Oil yellow II p. 71	As carcinogenic substance.
Chrysoidin Y p. 71	A general cytoplasm stain; a good substitute for Bismarck brown.
	Vital staining of pollen tubes.
	Vital staining of insects.
	Staining Golgi apparatus.

*The pages listed in this column refer to the present book.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Benzene-azo-α-naphthylamine p. 72	Vital stain for protozoa
Orange G p. 72	A valuable plasma stain in sections of tissue.
	Background stain for hematoxylin and other nuclear stains in botanical histology.
	As cytoplasm stain, in contrast to crystal violet and safranin; Flemming triple stain.
	For cytoplasm and red blood cells in Mallory's connective tissue stain; with anilin blue and acid fuchsin.
	For tissues; with methyl green and acid fuchsin in Ehrlich-Biondi-Heidenhain technic.
	Ehrlich's "tri-acid" stain for blood; combined with methyl green and acid fuchsin.
	Combined with gentian violet as Bensley's "neutral gentian"; for islands of Langerhans.
	Counterstain to thionin; in plant pathology.
	As constituent of Kornhauser's "Quad" stain.
Chromotrope 2R p. 73	Counterstain to celestin blue; in animal histology.
Azophloxine GA p. 73	As counterstain in neuropathology.
	Substitute for "xylidine ponceau" in Masson trichrome technic.
	Staining red blood cells in sections.
Sorbine red p. 74	Substitute for "xylidine ponceau" in Masson trichrome technic.
Azo acid blue B p. 76	Staining nervous tissue.
Nitrazine p. 76	As substitute for xylidine ponceau in Masson trichrome stain.
Sudan II p. 76	Fat stain for central nervous tissue, in supersaturated isopropanol solution.
Ponceau 2R p. 77	In Masson's trichrome tissue stain.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Oil brown D p. 77	As fat stain in Lillie's supersaturated isopropanol solution.
Bordeaux red p. 78	For cytoplasm, before staining with Heidenhain's hematoxylin.
	For sections of spleen, testis and liver; with methyl green and thionin.
	Substitute for "xylidine ponceau" in Masson trichrome technic.
Sudan R p. 78	Probably the dye employed in Weichherz's modification of the Kahn test for syphilis.
Janus green B p. 78	For vital staining of chondriosomes; also of fungi and protozoa.
	For sections of embryos; with neutral red.
	Sabins method for supravital stain for blood.
Janus black p. 80	Vital staining of pollen tubes.
Metanil yellow p. 80	In Masson's trichrome tissue stain.
Methyl orange p. 81	For determining reaction of cell sap in plants.
	With crystal violet in staining pollen tubes.
Prontosil p. 81	Vital stain for insects and plants.
Brilliant yellow S p. 82	As counterstain to carbol fuchsin in staining acid-fast bacteria; Doglio's method as interpreted by Margolena.
Orange I p. 82	With malachite green for sections of plant tissue.
Orange II p. 83	As cytoplasm stain; with eosin and azure C.
	In place of orange G in the Ehrlich-Biondi stain.
	For keratin in sections of skin.
	In Kalter's quadruple stain, with fast green FCF, safranin O and crystal violet.
Narcein p. 83	As component of some of Ehrlich's neutral stain mixtures.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Amaranth p. 84	For axis cylinders.
	Staining cells in tissue culture.
	In color photography.
Methyl red p. 84	As vital stain for protozoa.
Thiazine red R p. 85	As counterstain in animal histology, especially with hematoxylin, for skeletal and cardiac muscle.
Naphthol blue black p. 85	By Lillie as a stain for collagen.
Brilliant croceine p. 86	By Lillie, as substitute for "xylidine ponceau" in Masson's triple stain.
Sudan III p. 86	For fat tissues; Daddi-Herxheimer technic.
	For differentiating suberized and cutinized tissue in plants.
Sudan IV p. 87	For fat in tissues, Michaelis-Herxheimer technic, and Lillie's supersaturated isopropanol method.
Oil red O p. 88	For staining fat; as substitute for Sudan III or IV.
	With pyridin; for staining fat.
Oil red 4B p. 88	As fat stain, in Lillie's supersaturated isopropanol solution.
Biebrich scarlet, W. S. p. 89	A general plasma stain.
	For cytoplasm, after polychrome methylene blue or Unna's hematein.
	Mixed with alum hematoxylin; for staining tissue sections.
	In neutral stain combination with ethyl violet, by Bowie, for staining islets of Langerhans.
	As substitute for xylidine ponceau in Masson trichrome stain.
	With orange G and fast green FCF, for staining vaginal smears.
	In "picro-Mallory" combination of McFarlane.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Ponceau S p. 89	By Curtis (more recently Ruth; Leach) as an improvement over acid fuchsin in the Van Gieson connective tissue stain.
Orseillin BB p. 90	In contrast to crystal violet in staining a mycorrhizal fungus.
	By Maneval, as bacterial stain.
Bismarck brown Y p. 90	A general *plasma* stain, formerly much used. A good *mucin* stain. Good for *vital staining* and for *staining in bulk*.
	For bacteria, particularly in contrast to gentian violet in the Gram technic.
	Chromosomes in smear preparations of eggs.
Congo red p. 92	Background stain for hematoxylin and other nuclear stains.
	By Griesbach for axis cylinders.
	By Schaffer for sections of embryos.
	For staining plant mucin.
	By Klebs as reagent for cellulose.
	By Blackman as stain for Uredineae.
	By Matsuura for staining elastic tissue.
	By Benians as negative stain for bacteria.
	By Merton for protozoa.
	By Gutstein as vital stain for yeasts.
	By Bennhold for amyloid.
Erie garnet B p. 93	Mixed with azure A; by Geschikter as stain for frozen fresh tissue.
Trypan red p. 93	For vital staining.
	An early chemotherapeutic agent.
Benzopurpurin 4B p. 93	Plasma stain, especially in contrast to hematoxylin.

TABLE 3. *(Continued)*

Name of stain and page reference*	Application
Brilliant purpurin R p. 94	By Gutstein as vital stain for yeasts.
	By Lillie in connective tissue stain, with azofuchsin G and naphthol blue black.
Vital red p. 94	By Evans for vital staining.
Azo blue p. 95	Vital staining of Protozoa.
	As substitute for india ink in demonstrating bacterial capsules.
	Histochemical demonstration of magnesium (with quinalizarin and Titan yellow).
Dianil blue 2R p. 95	For vital staining.
Trypan blue p. 96	For vital staining.
Evans blue p. 96	For vital staining.
Vital new red p. 96	As vital stain.
Niagara blue 4B p. 97	By Varrelman for demonstrating vascular system of plants.
	Occasional use in general histology, and possibly in vital staining.
Chlorazol black E p. 97	In general histology.
	As auxiliary stain in plant cytology.
Sudan black B p. 98	As myelin stain.
	As general fat stain.
	As bacterial fat stain.
Bindschedler's green, p. 102	As oxidation-reduction indicator in study of metabolism.
Toluylene blue p. 102	As oxidation-reduction indicator.
Indophenol blue p. 103	By Herxheimer as fat stain.

TABLE 3. *(Continued)*

Name of stain and page reference*	Application
	For chromatin and mucin in general histology; and as general nuclear stain.
	For frozen sections of fresh tissues.
	For amyloid (colored blue), mast cells and mucin (red).
Thionin p. 104	With methyl green and Bordeaux red, by Gråberg for sections of spleen, testis and liver.
	By Stoughton for staining bacteria and fungi in plant tissue; with orange G as counterstain.
	End-point staining of Nissl granules.
	By Schmorl for staining bone sections.
Azure C p. 107	By French as nuclear stain; with eosin and orange II.
	As nuclear stain; preceding eosin or following phloxine.
	In neutral stain combination with eosin; for staining blood, protozoa, tissue, etc.
Azure A p. 109	Constituent of MacNeal's tetrachrome stain; for staining blood.
	By Custer for staining bone marrow.
	By Kinyoun in a modification of the Ponder diphtheria stain.
	By Jordan and Heather for Negri bodies.
Azure B p. 110	Important constituent of blood stains, especially Lillie's modification of Giemsa stain.
	A widely used nuclear stain in general histology (zoological), with many special applications.
	In polychrome methylene blue; as tissue stain.
	A favorite bacterial stain, used for many special purposes, as in examination of milk and diagnosis of diphtheria.
	By Mallory as nuclear stain, in contrast to phloxine, on pathological or other histological material.
Methylene blue p. 111	Staining small animals *intra vitam*.
	Vital staining of nervous tissue.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Methylene blue (*Cont.*)	Mixed with basic fuchsin; for nervous tissue, in diagnosis of rabies.
	For staining blood; in combination with eosin.
	With eosin, by Levine as indicator in bacteriological media, for differentiating colon and aerogenes organisms.
Methylene violet p. 114	Constituent of MacNeal's tetrachrome blood stain.
	Counterstain to alizarin red S for calcium deposits.
Methylene green p. 115	For wood and fixed chromatin, in plant sections. (Does not stain fresh chromatin.)
	By Lison as vital stain for insects.
Toluidine blue O p. 115	A metachromatic nuclear stain often useful in place of thionin or methylene blue. (Pappenheim's panchrome stain.)
	Diagnosis of diphtheria. (Albert; Kinyoun)
	As general tissue stain.
	Histochemical agent for determining thymonucleic acid.
New methylene blue N, p. 116	By Van Wijhe for cartilage in amphibia.
Brilliant cresyl blue, p. 117	For blood; to bring out platelets and reticulated cells.
	As a vital stain, to indicate O–R potential.
	Staining protozoa and nematodes in animal cytology.
Gallocyanin p. 119	Nuclear stain for tissue.
	Counterstain to protargol, for nervous tissue.
Gallamin blue p. 119	Nuclear stain; used in form of iron lake.
Celestin blue B p. 120	Nuclear stain for tissue.
	In Lendrum and MacFarlane's modification of Mallory connective tissue stain.
Lacmoid p. 120	By Tsvett as microchemical reagent for detection of callose.
	With martius yellow in a stain for pollen tubes.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Nile blue sulfate p. 121	Lorain Smith's method to distinguish between fatty acids and neutral fat.
	Supravital stain for embryos.
	Vital stain for hydrae, protozoa, and yeasts.
	For amphibian eggs.
	In bacteriological media; to show lipolysis.
	Staining sections of bone.
	Histochemical studies of Golgi apparatus.
Cresyl violet p. 122	For fixed tissue, (especially of tumors).
	For nervous tissue.
	For vital staining of blood.
	For fresh tumor tissue.
New blue R p. 124	Staining fat drops in bacteria.
Resazurin p. 124	As oxidation-reduction indicator, particularly for milk tests.
Neutral red p. 126	For histological tissue (of embryos); contrast stain to Janus green.
	Nuclei, especially of blood cells, and Nissl granules of nerve cells, stained *intra vitam*.
	"Vital staining" of blood, (i.e., fresh in moist chamber); also of fresh gonorrhoeal pus.
	As indicator in bacteriological culture media; for distinguishing colon from typhoid organisms, and other similar purposes.
	With light green, by Twort as stain for parasites in tissue.
	Histochemical reagent for demonstrating hydrolysis of fats.
Neutral violet p. 128	Used in study of chromolysis by Unna.
Pheno-safranin p. 128	By Moore for colonies of bacteria and fungi.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Safranin O p. 129	A widely used nuclear stain.
	For tissues of vascular plants, in combination with variety of contrast stains. Cutinized, suberized, and lignified tissue. Spore coats. Protein.
	In Flemming's triple stain, for chromatin and other nuclear elements, in conjunction with gentian violet; orange G as contrast stain.
	For plant chromatin; with light green or anilin blue W.S.
	For bacteria, particularly in contrast to gentian violet in the Gram technic.
Amethyst violet p. 131	Nuclear dye in certain triple staining procedures.
Azocarmine G p. 132	As tissue stain; especially preceding Mallory's anilin-blue-orange-G mixture. Heidenhain's "Azan stain."
	As counterstain to a resorcin gentian violet.
	With iron-hematoxylin and naphthol green B; by Mollier as tissue stain.
Magdala red p. 133	By Kulschitzky for elastic tissue.
	By Flemming as nuclear stain.
Indulin, spirit sol. p. 134	For negative staining of bacteria.
Indulin, water sol. p. 134	Some indulin used in counterstaining bulk tissue.
Nigrosin, water sol. p. 134	For central nervous tissue; alone or with other stains.
	For tissue of pancreas; following hematoxylin.
	Pfitzer's picronigrosin for chromatin.
	With basic fuchsin in staining bacterial spores (Dorner).
Auramin O p. 139	By Fischel for vital staining of salamander larvae.
	By Vinassa for plant sections.
	With cresyl violet; by Kisser as tissue stain.
	By Richards and Miller for fluorescent staining of acid-fast bacteria.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Malachite green p. 141	In combination with acid fuchsin and martius yellow; Pianese's stain for cancer tissue.
	For host tissue in plants infected with fungi; used in the Pianese combination.
	By V. Beneden for Ascaris eggs.
	By Petroff for erythrocytes.
	Contrast stain, following borax carmine.
	By Shaeffer and Fulton for bacterial spores; with safranin as counterstain.
	To replace methyl green in Pappenheim stain.
	By Bank as vital stain for epidermis of onion.
	Reagent for bacterial polysaccharides (Chapman and Lieb).
	Bacteriostatic or amebicidic agent.
Brilliant green p. 142	Indicator in bacteriological media for differentiating organisms of colon, typhoid and dysentary groups (Krumwiede).
	For inhibiting *B. coli* in stools.
	Enrichment of the typhoid organism in broth cultures.
Light green SF yellowish p. 143	A general plasma stain.
	By Benda for spermatozoa; with safranin.
	For cellulose walls in vascular plant tissue; contrast to safranin.
	For general histological tissues; contrast to hematoxylin.
	Following safranin; in cytology.
	With neutral red; by Twort for parasites in tissues.
	Mixed with acid fuchsin, for staining pollen tubes. (Buchholz).
Fast green FCF p. 144	As substitute for light green SF yellowish in histology and cytology.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Fast green FCF (*Cont.*)	A widely used plasma stain in both animal and plant microtechnic.
	With safranin, methyl violet, and orange G, for plant tissue.
	As bacterial stain.
	As constituent of Kornhauser's "Quad" stain.
	In a modification of the Van Gieson connective tissue stain.
Basic fuchsin p. 146	A powerful nuclear stain; with various green and blue contrast stains.
	For mucin, fuchsinophile granules; for nuclear elements of central nervous system.
	Mixed with methylene blue; for staining Negri bodies in nerve cells.
	Preceding crystal violet; for staining bacilli in tissues.
	For elastic tissue.
	By Williams for Negri bodies.
	General bacterial staining, both in smears amd in sections.
	Ziehl-Neelsen technic for staining tubercle organism; diagnosis by acid-fast property.
	In bacteriological culture media for differentiating colon and typhoid organisms—Endo medium.
	As histochemical reagent for recognition of nucleic acids and other polysaccharides—the Feulgen stain, HIO_4-Schiff reaction, etc.
Acid fuchsin p. 153	A widely used plasma stain.
	In combination with picric acid; the Van Gieson connective tissue stain.
	In combination with anilin blue and orange G; the Mallory connective tissue stain.
	In combination with methyl green and orange G; Ehrlich-Biondi-Heidenhain differential stain for sections and blood smears.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Acid fuchsin (*Cont.*)	In combination with methyl green and orange G; Ehrlich's "Tri-acid stain" for blood smears.
	For cortex, pith, cellulose walls, etc., in vascular plants.
	Pianese stain for cancer tissue; with malachite green and martius yellow.
	Same technic adapted for staining fungus mycelium in infected plants.
	Bensley-Cowdry stain for mitochondria; with methyl green.
	As Andrade indicator; especially in bacteriological media.
Hofmann's violet p. 155	For mast cells—Ehrlich.
	For amyloid in sections of tissue.
Methyl or Gentian violet p. 156	A powerful nuclear stain.
	A very valuable stain in plant histology, particularly for the achromatic nuclear structures.
	For nuclear structures in conjunction with safranin; orange G as contrast stain—Flemming triple stain.
	For fibrin and neuroglia in fresh tissue.
	For amyloid; in frozen sections of fresh tissue.
	Combined with orange G—Bensley's "neutral gentian", for demonstrating islands of Langerhans.
	In Petroff's egg medium for cultivation of tubercle organism.
	For bacteriostatic properties.
Crystal violet p. 160	For staining bacteria in tissues—Gram-Weigert method.
	The Gram stain for bacteria; generally with alcoholic differentiation to make it selective, and some red or brown dye as counterstain.
	For diphtheria organism and bacterial capsules.
	Gram stain for tissues.
	Benda's stain for mitochondria; with alizarin red.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Crystal violet (*Cont.*)	Jackson's stain for lightly lignified walls in plants; with erythrosin.
	Following basic fuchsin and picric acid; MacCallum's stain for influenza bacilli in tissues.
	A compound of this dye with bismuth ("bismuth violet") used for its bacteriostatic properties.
	With iodine, in plant cytology.
Ethyl violet p. 161	In combination with Biebrich scarlet; for staining the islets of Langerhans—Bowie.
	For nervous tissue.
	For spirochaetes in blood.
Methyl green p. 162	An excellent nuclear stain.
	In weak acetic acid solution, for fresh chromatin.
	For lignified xylem in plants; with acid fuchsin.
	As cytoplasm stain; following acid fuchsin and picric acid.
Ethyl green p. 164	In combination with acid fuchsin and orange G; Ehrlich's "Tri-acid stain" for blood smears.
	In combination with acid fuchsin and orange G; stains nuclei in sections and in blood—Ehrlich-Biondi-Heidenhain.
	Bensley-Cowdry stain for chromatin; in contrast to acid fuchsin, which stains the mitochondria.
	In combination with pyronin; Pappenheim-Saathof stain for gonococcus, and for mast cells.
Iodine green p. 164	As nuclear stain—Griesbach.
	For mucin and amyloid.
	For lignified xylem in plant sections; with acid fuchsin.
	For chromatin in plant tissue; with acid fuchsin for nucleolus and spindle fibers.
	For nervous tissue; with acid fuchsin and picric acid.
	For blood; with acid fuchsin.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Iodine green (*Cont.*)	In a differential medium for the gonococcus.
Spirit blue p. 166	Counterstain to carmine; especially for embryonic tissues.
	Counterstain to carmine in bulk staining of small animals and embryos.
	Color base used by Knaysi in determining hydrolysis of fats.
Victoria blue 4R p. 167	For granules in virus diseases (claimed to be the virus).
	As stain for bacterial flagella.
	By Lipp as stain for spirochaetes, especially *Treponema pallidum*.
Methyl blue p. 167	A valuable counterstain in animal histology.
	Mann's stain for nerve cells; with eosin.
	Combined with picric acid, as tissue stain; followed by carmalum or safranin.
	For unfixed tissue.
	For negative staining of bacteria.
	A vital stain for protozoa—Monné.
Anilin blue water sol., p. 168	A good plasma stain, used particularly in nervous tissue and cartilage.
	In Mallory's connective tissue stain; with orange G and acid fuchsin.
	As cytoplasm stain, preceding safranin.
	For sections of epithelium; with orcein—Unna.
	For nervous tissue.
	In bacteriological media for the isolation of the crown gall organism.
Isamine blue p. 170	As vital stain by intravenous injection.
Patent blue V p. 170	For spirochaetes in blood.
	For vital staining.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Patent blue (*Cont.*)	As an acid-base and oxidation-reduction indicator.
Xylene cyanol FF p. 171	As an intracellular indicator.
	In the study of permeability of tissue membranes.
	Vital staining of cells in tissue culture.
Rosolic acid p. 172	With methylene blue in a decolorizing solution following basic fuchsin; for tubercle organism—Pappenheim.
Victoria blue R p. 173	For spirochaetes in blood.
	Vital stain for yeasts.
Victoria blue B p. 174	For spermatozoa in testicle.
	For nervous tissue.
	In form of eosinate, for frozen fresh tissue.
Night blue p. 174	By Hallberg as stain for acid-fast bacteria.
Wool green S p. 175	By Lillie as substitute for fast green FCF in the Masson trichrome stain.
Acridine red 3B p. 178	With methyl green; by Gomori for staining tissue to demonstrate calcium despoits.
Pyronin Y p. 178	In Scudder's combined Gram-Pappenheim stain for gonorrhoeal pus.
	In same procedures as pyronin B.
Pyronin B p. 180	In combination with methyl green; Pappenheim-Saathof stain for bacteria (especially in organic liquids), mast cells and other basophile elements.
	By Ehrlich in combination with narcein and methyl green or methylene blue, for blood, etc.
	For bacteria; sometimes used as counterstain to gentian violet in the Gram technic.
	By Monné for staining mitochondria.
	By Huddleson as bacteriostatic agent in distinguishing species of *Brucella*.
Rhodamine B p. 181	With osmic acid; by Griesbach to fix and stain blood simultaneously.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Rhodamine B (*Cont.*)	As plasma stain, in contrast to methyl green or methylene blue.
	By Ehrlich as a component of certain "neutral" stain mixture; for blood, etc.
	Mixed with methylene blue, for glandular tissue—Houcke.
	In fluorescence microscopy.
	In microchemical estimation of antimony.
Rhodamine 6G p. 182	In fluorescence microscopy.
	In studies of Golgi apparatus.
Violamine R p. 182	By Lillie as stain for collagen.
Fast acid blue R p. 183	By Romell as stain for bacteria in soil.
Fluorescein p. 186	In fluorescence microscopy.
	In culture media for fungi.
Eosin Y p. 187	Very useful cytoplasm stain.
	For algae and fungi; counterstain to hematoxylin, etc.
	In combination with methyl blue; Mann stain for sections of tissues.
	By List as cytoplasm stain; preceding methyl green.
	For sputum; preceding methylene blue—Teichmüller.
	In combination with methylene blue; for blood smears.
Ethyl eosin p. 190	Counterstain following Delafield's hematoxylin in general animal histology.
	Stain for Negri bodies in smears or sections of nervous tissue.
Eosin, bluish p. 191	Counterstain, especially following Mayer's hemalum.
	By Lillie as constituent of neutral stain for tissue, compounded with azure A or toluidine blue O.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Mercurochrome 220 p. 192	Substitute for eosin.
	Fungicidal agent for treating amphibian embryos.
	Counterstain to malachite green in Wirtz spore stain for bacteria.
Erythrosin p. 193	Cytoplasm stain for nerve cells; preceding methylene blue.
	Counterstain for sections of vascular plants; in contrast to hematoxylin, gentian violet, etc.
	Stain for bacteria in dried smears of soil suspensions.
	By Jackson as counterstain to crystal violet in plant histology.
	By Gellhorn as vital staining of sea urchin eggs.
Phloxine p. 195	For Negri bodies.
	For staining algae.
	Combined with various other acid dyes; used by Unna in studying chromolysis.
	By Mallory as cytoplasm stain in contrast to methylene blue, on pathological or other histological material.
Rose bengal p. 197	Cytoplasm stain, following hematoxylin.
	Stain for bacteria, especially those forming slime; also for dried smears of soil suspensions.
	Negative staining of bacteria.
	For spirochaetes in blood.
	In test for liver function—Delprat and Stowe.
	As fluorochrome in study of fats.
Brom cresol purple p. 204	Hydrogen-ion indicator, used as vital stain.
Brom thymol blue p. 204	Hydrogen-ion indicator, used as vital stain.
	For staining fungi in roots.

TABLE 3. (*Continued*)

Name of stain and page reference*	Application
Acridine yellow p. 206	Four dyes used as fluorochromes, for example in insect histology by Metcalf and Patton.
Corphosphine O p. 207	
Acridine orange NO p. 207	
Rivanol p. 207	
Acriflavine p. 208	With gentian violet; for bacteriostatic properties.
	In fluorescence microscopy.
Proflavine p. 208	For bacteriostatic properties.
Atabrine p. 209	By Lewis and Goland in vital staining and retardation of tumors.
	In fluorescence microscopy.
Phosphine p. 209	Following methylene blue; by Schumacher as microchemical reagent for nucleo-proteids.
	In fluorescence microscopy.
Rheonine A p. 210	In fluorescence microscopy.
Saffron p. 211	In Masson's trichrom tissue stain.
	Staining yellow fever lesions in liver.
Indigo carmine p. 212	As plasma stain in contrast to carmine, either with it or following it; also with basic fuchsin or other nuclear stain.
	With acid fuchsin, by Petragnani as stain for Negri bodies.
	With hematoxylin, for vaginal smears.
	In plant cytology, with picric acid and basic fuchsin.
	For urethral injection in studies of glomerular excretion.
Berberine p. 212	In fluorescent microscopy.

TABLE 3. (*Continued*)

Name of stain, mordant, etc. Also page ref.*	Application
Cochineal, p. 213	For general discussion see Krause (1926–7), p. 394–8.
Alone, aqueous	Only for objects containing iron, aluminum, etc.
Alone, alcoholic	Mayer's old formula. Especially for arthropod tissue.
Tincture, (with $CaCl_2$ & $AlCl_3$)	By Mayer for sections and staining in bulk. Used like paracarmine (see below).
Alum-cochineal	For staining in bulk; not suited for sections. (Stains nuclei violet red, blood and muscle cells orange; colors cytoplasm weakly.)
	With hematoxylin, by Reynolds for staining trematodes and nematodes.
Chromalum cochineal	By Hansen for sections.
Iron alum cochineal (i.e. following iron alum)	For staining in bulk for photographing; also for sections; brings out nuclei, blood in tissues, and muscle striations.—Spuler.
Ferri-cochineal (i.e. with iron alumn)	By Hansen for sections.
Carmine, p 214	For general discussion see Krause (1926–7), p. 260–8.
Aceto-carmine	As nuclear stain for sections; for fresh chromosomes in smear preparations.—Schneider.
Iron-aceto-carmine	By Belling as stain for chromosomes in smears of anthers
Ammonia carmine	For sections, as by Ranvier and Hoyer.
	For injection, by Van Wijhe.
	By Best for staining glycogen in sections.
Magnesia carmine	By Mayer for sections, and for bulk staining.
Borax carmine (alcoholic)	A much used stain for sections, following Grenacher, and Mayer.
Hydrochloric carmine (alcoholic)	By Mayer for sections, and for bulk staining.
Alum carmine	By Grenacher as nuclear stain for sections.

TABLE 3. (*Continued*)

Name of stain, mordant, etc. Also page ref.*		Application
	Lithium carmine	By Orth as nuclear stain for sections.
	With $AlCl_3$ "Muci-carmine"	By Mayer for mucin, in sections.
	Iron carmine	For sections.
	Picro-carmine (with picric acid)	For double staining effect in sections, particularly of nervous tissue. Nuclei red; cytoplasm yellow.
Carminic acid, p. 214		For general discussion, see Krause (1926–7), p. 268–71.
	Carmalum (with alum)	By Mayer as nuclear stain for sections.
		Prepared with glycerin; by Rawitz for sections.
	Muci-carminic acid (acid, with $AlCl_3$)	By Rawitz for mucin, in sections.
	Para-carmine (with $AlCl_3$ and $CaCl_2$)	By Mayer for sections and staining in bulk; ordinarily stains cytoplasm as well as nuclei.
	With iron	Following treatment with iron chloride; for sections and staining in bulk—Weigert; Van Wellheim.
Orcein, p. 216		By Unna, in alcoholic solution; for elastic tissue; first proposed by Taenzer.
		By Israel, dissolved in acetic acid solution. Stains nuclei blue, cytoplasm red.
		By Moll in HCl solution; for sections of embryos.
		By Unna, for connective tissue; following polychrome methylene blue.
		By Unna, for plasma fibrils of epithelium; following anilin blue.
		By Mollier, with naphthol green B and iron-hematoxylin; as tissue stain.
		With acid alizarin blue, orange G, and fast green FCF; as tissue stain.—Kornhauser's "Quad" stain.
		By LaCour in a stain-fixative for cytology.

TABLE 3. (*Continued*)

Name of stain, mordant, etc. Also page ref.*		Application
Brazilin, p. 218		By Mayer, with alum, as a nuclear stain (like hemalum). Called brazalum.
		By Hickson, as nuclear stain, following iron alum.
		By Belling for staining chromosomes in smears of anthers.
		By Shaffer for distinguishing different types of fibres in paper.
Hematoxylin and hematein, p. 219		For general discussion, see Krause (1926–7), p. 958–98.
	Alone	As nuclear stain for plant sections.
		As reagent for iron and copper.
	With aluminium (alum-hematoxylin)	By Delafield as nuclear stain for tissues. For cellulose walls of plants.
		By Ehrlich as nuclear stain for tissues.
	Hemalum (hematein with alum)	By Mayer as nuclear stain for tissues.
	Hemacalcium (hematein with $AlCl_3$ and $CaCl_2$	By Mayer as nuclear stain for tissues.
	Muci-hematein (with $AlCl_3$ and glycerin)	By Mayer as stain for mucin.
	With chromium	An early technic for tissue staining by M. Heidenhain; still used.
		Modifications by Apathy, Hennegin, Hansen, Schultz; for general tissue staining.
		By Weigert for nervous tissue.
	Following an iron salt	M. Heidenhain's method. One of the most useful present histological and cytological stains, both in botany and zoology.
		A valuable nuclear stain for sections—Weigert.
		Modifications by Bütschli, Hansen, Kaiser for various special purposes.

TABLE 3. (*Continued*)

	Name of stain, mordant, etc. Also page ref.*	Application
Hematoxylin	Following copper	By Benda for studying spermatogenesis.
		By Bensley for chromosomes and mitochondria.
	With phosphomolydbic acid	By Mallory for central nervous system.
	With phosphotungstic acid	A very valuable pathological stain originated by Mallory, especially for fibrils in tumor tissue.
	With vanadium	By M. Heidenhain for general tissue.
	Double staining	Followed by picric acid and acid fuchsin. Van Gieson's connective tissue stain.
		Sometimes followed by picric acid, or following eosin, orange G, safranin or acid fuchsin.
	Alizarin p. 225	As vital stain for protozoa.
		To distinguish cows' milk from goats' milk (Geyer).
	Alizarin red S p. 226	By Benda for chromatin; in combination with crystal violet (chromatin brown, mitochondria violet).
		For section of nervous tissue.
		Vital stain for nerve tissues.
		Dawson's method for gross staining of skeletons in embryos and small animals.
		Chromosome stain in plant cytology.
	Purpurin p. 227	A nuclear stain.
		Reagent for detecting insoluble calcium salts in protoplasm.
	Quinalizarin p. 227	Histochemical demonstration by Broda of magnesium (with azo blue, and Titan yellow.)
	Carycinel red p. 228 Coccinel red p. 228	Two dyes named by Lillie and proposed by him as fat stains in the saturated isopropanol technic.
	Oil blue NA p. 228	Stain for rubber in plant sections.
		Stain for fat in animal tissue.

TABLE 3. (*Concluded*)

Name of stain, mordant, etc. Also page ref.*	Application
Acid alizarin blue GR p. 229	As tissue stain by Buzaglo; with orcein and gallocyaine.
Acid alizarin blue BB p. 229	In fluorescence microscopy.
Alkanet p. 229	Stain for rubber in plant sections.
Geranine G p. 230	In fluorescence microscopy.
Primulin p. 230	As vital stain, in fluorescence microscopy.
Titan yellow G p. 231	As vital stain, in fluorescence microscopy.
	As indicator for determining magnesium in body fluids.
Thioflavine T p. 231	As fluorochrome, for example by Metcalf and Patton in insect histology.
Thioflavine S p. 331	As fluorochrome in plant pathology and insect histology.
Pinacyanol p. 332	By Proescher for frozen fresh tissue.
	By Hetherington as supravital stain for mitochondria in blood.
Diazonium and Tetrazonium salts p. 232–5 Tetrazolium salts p. 236–7	Colorless salts which are converted into colored azo compounds in the presence of certain products of enzyme activity. This property gives them value as histochemical reagents.

Table 4. List of Biological Stains on Certification Basis, January 1, 1953

Alizarin red S
Anilin blue, water soluble
Auramine O
Azocarmine G
Azure A
Azure B
Azure C
Bismarck brown Y
Brilliant cresyl blue
Brilliant green
Carmine
Chlorazol black E
Congo red
Cresyl violet
Crystal violet
Eosin, bluish
Eosin, yellowish
Erythrosin B
Ethyl eosin
Fast green FCF
Fuchsin, acid
Fuchsin, basic
Giemsa stain
Hematoxylin
Indigo carmine
Janus green B
Jenner's stain
Light green S. F. yellowish
Malachite green
Methyl green
Methyl orange
Methyl violet 2B
Methylene blue chloride
Methylene blue thiocyanate
Methylene violet
Neutral red
Nigrosin
Nile blue A
Orange G
Orange II
Orcein
Phloxine
Pyronin B
Pyronin Y
Resazurin
Rose bengal
Safranin O
Sudan III
Sudan IV
Sudan black B
Tetrachrome stain (MacNeal)
Thionin
Toluidine blue O
Wright's stain

Table 5. List of Biological Stains Grouped According to the Field in Which Used*

ANIMAL HISTOLOGY

Nuclear stains (basic)	
Janus green B	**Basic fuchsin**
Thionin	Hofmann violet
Azure A	Iodine green
Azure C	**Crystal or methyl violet**
Methylene blue	**Cochineal and carmine**
Toluidine blue O	Orcein
Cresyl violet	Brazilin
Safranin O	**Hematoxylin**
Magdala red	**Neutral red**
Auramine O	
	Nigrosin
Cytoplasm stains (acid)	Malachite green
Picric acid	**Light green SF yellowish**
Orange G	Fast green FCF
Bordeaux red	**Acid fuchsin**
Fast yellow (bone tissue)	Methyl blue
Methyl orange (for keratin in skin)	**Anilin blue W. S.**
Amaranth (nervous tissue)	Rhodamine B
Biebrich scarlet W. S.	Eosin Y
Bismarck brown Y	Eosin B
Chrysoidin Y	Ethyl eosin
Congo red	Phloxine
Benzopurpurin 4B	Erythrosin (nervous tissue)
Alizarin red S (for nervous tissue)	Indigo carmine

*In this table the stains in bold face type are those of widest application. When a stain is specified for practically only one purpose that purpose is mentioned in parenthesis; the stains not so designated are of fairly general application in the particular field under which they are listed.

Fat stains
- **Sudan III**
- **Sudan IV**
- Sudan black B
- Oil red O
- Nile blue sulfate

Vital stains
- Bismarck brown Y
- Chrysoidin Y
- Trypan red
- Benzopurpurin **4B**
- Trypan blue
- Vital red
- Dianil blue **2B**
- **Methylene blue**
- Thionin
- Toluidine blue O
- Nile blue sulfate
- Brilliant cresyl blue
- **Neutral red**
- Safranin O
- **Janus green B**
- Crystal violet
- Methyl violet

FLUORESCENT MICROSCOPY

- Acid alizarin blue BB
- Acid fuchsin
- Acridine orange NO
- Acridine yellow
- Acriflavine
- **Auramine O**
- Coriophosphine O
- Fluorescein
- Berberine sulfate
- Geranine G
- Phosphine
- Primulin
- Rheonin A
- Rhodamine B and 6G
- Rivanol
- Rose Bengal
- Thioflavine S
- Titan yellow G

PLANT HISTOLOGY

Stains for cell walls, etc.
- Acid fuchsin
- Aniline blue W.S.
- Bismarck brown Y
- Congo red
- Crystal or methyl violet
- Erythrosin
- Fast green FCF
- Hematoxylin
- Iodine green
- **Light green SF yellowish**
- **Methyl green**
- Methylene blue
- Methylene green
- **Safranin O**
- Sudan IV

Nuclear stains (basic)
- Auramine O
- **Cochineal** and **carmine**
- **Crystal** or **methyl violet**
- Basic fuchsin
- **Hematoxylin**
- Iodine green
- **Safranin O**
- Thionin

Cytoplasm stains (acid)
- **Acid fuchsin**
- Anilin blue W.S.
- Congo red (mucin)
- **Eosin Y**
- Erythrosin
- Fast green FCF
- Malachite green } For host tissue
- Martius yellow } in fungus disease
- Methyl orange
- Nigrosin
- **Orange G**
- Phloxine

CYTOLOGY

General nuclear stains (basic)
- Thionin
- **Methylene blue**
- Toluidine blue O
- Magdala red
- Basic fuchsin
- **Crystal** or **methyl violet**
- **Methyl green**
- **Carmine**
- Orcein
- **Hematoxylin**

Special chromatin stains
- Alizarin red S
- Thionin
- Methylene green
- **Safranin O**
- Iodine green
- **Hematoxylin**
- **Crystal violet**
- Brasilin
- **Carmine**

Cytoplasm stains (acid)

Picric acid
Orange G
Light green SF yellowish
Fast green FCF
Bordeaux red
Methyl orange
Acid fuchsin
Eosin Y

Stains for cell inclusions (mitochondria, etc.)

Aurantia
Janus green B
Hematoxylin
Acid fuchsin
Crystal violet

PATHOLOGY AND BACTERIOLOGY

Nuclear stains (basic)

Thionin
Azure A
Azure C
Methylene blue
Toluidine blue O
Safranin O
Cresyl violet
Basic fuchsin
Hofmann violet
Iodine green
Crystal or **methyl violet**
Pyronin
Cochineal and **carmine**
Orcein
Hematoxylin

Cytoplasm stains (acid)

Picric acid
Martius yellow (for cancer tissue)
Orange G
Methyl orange
Amaranth (for nervous tissue)
Biebrich scarlet W. S.
Congo red
Alizarin red S
Neutral red
Nigrosin
Malachite green
Light green SF yellowish
Acid fuchsin
Methyl blue
Anilin blue, W S.
Eosin Y
Eosin B
Ethyl eosin
Phloxine
Erythrosin

Blood stains and their constituents

Giemsa stain
Wright stain
Tetrachrome stain
Methylene blue (basic)
Azure A (basic)
Eosin Y (acid)

Fat stains

Sudan IV
Nile blue sulfate
Oil red O
Sudan black B

Bacterial stains

Bismarck brown (Gram counterstain)
Thionin
Methylene blue
Toluidine blue O
Safranin O (Gram counterstain)
Basic fuchsin
Gentian violet (including crystal and methyl violets)
Nigrosin
Methyl green (constituent of Pappenheim stain)
Pyronin (same)
Erythrosin
Rose bengal

Used in bacteriological media

Neutral red
Basic fuchsin
Acid fuchsin
Brilliant green
Methylene blue
Eosin Y

Table 6. Dye Solubilities at 26°C. (Purified Samples).

Based on data obtained by Holmes (*1927, 1928, 1929*).

Note: With a few exceptions (the iodides, the magnesium and calcium salts) these figures are for commercial dyes recrystalized from water or aqueous-alcoholic mixtures. Commercial dyes are often considerably less soluble; see Table 7.

Colour Index Number	Name of dye	Per cent soluble in	
		Water	95% alcohol
1027	Alizarin	nil	0.125
1034	Alizarin red S	7.69	0.15
40	Alizarol orange G	0.40	0.57
36	Alizarol yellow GW	25.84	0.04
184	Amaranth	7.20	0.01
847	Amethyst violet	3.12	3.66
655	Auramin O	0.74	4.49
12	Aurantia	nil	0.33
146	Azo acid yellow	2.17	0.81
88	Azo Bordeaux	3.83	0.19
448	Benzopurpurin 4B	——	0.13
280	Biebrich scarlet	——	0.05
332	Bismarck brown R	1.10	0.98
331	Bismarck brown Y	1.36	1.08
252	Brilliant crocein	5.04	0.06
29	Chromotrope 2R	19.30	0.17
21	Chrysoidin R	0.23	0.99
20	Chrysoidin Y	0.86	2.21
370	Congo red	——	0.19
89	Crystal ponceau	0.80	0.06
681	Crystal violet (chloride) } gentian	1.68	13.87
	Crystal violet (iodide) } violets	0.035	1.78
——	Cresyl violet (N. A. Co.)	0.38	0.25
715	Cyanol extra	1.38	0.44
771	Eosin B (Na salt)	39.11	0.75
768	Eosin Y† (Na salt)	44.20	2.18
	Eosin Y† (Mg salt)	1.43	0.28
	Eosin Y† (Ca salt)	0.24	0.09
	Eosin Y† (Ba salt)	0.18	0.06
130	Erika B	0.64	0.17
254	Erythrin X	6.41	0.06
773	Erythrosin† (Na salt)	11.10	1.87
	Erythrosin† (Mg salt)	0.38	0.52
	Erythrosin† (Ca salt)	0.15	0.35
	Erythrosin† (Ba salt)	0.17	0.04
770	Ethyl eosin	0.03	1.13
——	Fast green FCF	16.04	0.35
176	Fast red A	1.67	0.42
16	Fast yellow	18.40	0.24
766	Fluorescein (color acid)	0.03	2.21
	Fluorescein (Na salt)	50.20	7.19
	Fluorescein (Mg salt)	4.51	0.35
	Fluorescein (Ca salt)	1.13	0.41
	Fluorescein (Ba salt)	6.54	0.56
	Fuchsin, basic:		
676	Pararosanilin (chloride)	0.26	5.93
——	Pararosanilin (acetate)	4.15	13.63
——	Rosanilin (chloride)	0.39	8.16
678	New fuchsin (chloride)	1.13	3.20
——	Gentian violet (see crystal or methyl violet)		

†The color acids of these dyes (not listed here) are practically insoluble in water.

Colour Index Number	Name of dye	Per cent soluble in	
		Water	95% alcohol
666	Guinea green B	28.40*	7.30
1180	Indigo carmine	1.68	0.01
133	Janus green	5.18	1.12
670	Light green SF yellowish	20.35	0.82
657	Malachite green (oxalate)	7.60	7.52
9	Martius yellow, Na salt	4.57	0.16
	Martius yellow, Ca salt	0.05	1.90
138	Metanil yellow	5.36	1.45
142	Methyl orange	0.52	0.08
	Methyl orange (acid)	0.015	0.015
680	Methyl violet (Gentian violet)	2.93	15.21*
922	Methylene blue ($ZnCl_2$ double salt)	2.75	0.05
	Methylene blue (chloride)	3.55	1.48
	Methylene blue (iodide)	0.09	0.13
924	Methylene green	1.46	0.12
10	Naphthol yellow G	8.96	0.025
152	Narcein	10.02	0.06
825	Neutral red (chloride)	5.64	2.45
	Neutral red (iodide)	0.15	0.16
826	Neutral violet	3.27	2.22
927	New methylene blue N	13.32*	1.65
728	New Victoria blue R	0.54	3.98
520	Niagara blue 4B	13.51	nil
914	Nile blue 2B	0.16	0.62
73	Oil red O	nil	0.39
150	Orange I	5.17	0.64
151	Orange II	11.37	0.15
27	Orange G	10.86	0.22
714	Patent blue A	8.40	5.23
774	Phloxine† (Na salt)	50.90*	9.02
	Phloxine† (Mg salt)	20.84	29.10
	Phloxine† (Ca salt)	3.57	0.45
	Phloxine† (Ba salt)	6.01	1.17
7	Picric acid	1.18	8.96
28	Ponceau 2G	1.75	0.21
186	Ponceau 6R	12.98	0.01
741	Pyronin B (iodide)	0.07	1.08
739	Pyronin Y	8.96	0.60
148	Resorcin yellow	0.37	0.19
749	Rhodamine B	0.78	1.47
750	Rhodamine G	1.34	6.31
779	Rose bengal† (Na salt)	36.25	7.53
	Rose bengal† (Mg salt)	0.48	1.59
	Rose bengal† (Ca salt)	0.20	0.07
	Rose bengal† (Ba salt)	0.17	0.05
841	Safranin	5.45	3.41
689	Spirit blue	nil	1.10
24	Sudan I	nil	0.37
248	Sudan III	nil	0.15
258	Sudan IV	nil	0.09
920	Thionin	0.25	0.25
925	Toluidine blue O	3.82	0.57
690	Victoria blue 4R	3.23	20.49
569	Victoria green 3B	0.04	2.24
8	Victoria yellow	1.66	1.18

*These figures are grams per hundred grams of saturated solution (the others being grams per hundred milliliters).

†The color acids of these dyes (not listed here) are practically insoluble in water.

Table 7. Solubilities of Certain Certified Commercial Stains.

Data obtained in the laboratory of the American Pharmaceutical Association, and published with the permission of the Association

Dye	Per cent soluble in									
	Water					95% alcohol				
	Pure* dye	Commercial samples†				Pure* dye	Commercial samples†			
		A	B	C	D		A	B	C	D
Alizarin red S	7.69	4.8	4.25	4.15	—	0.15	0.5	0.26	0.26	—
Aniline blue W. S.	—	3.7	4.8	4.8	—	—	0.48	0.17	0.31	—
Azure A	—	4.15	—	—	—	—	0.68	—	—	—
Bismarck Brown Y	1.36	1.23	3.13	2.44	—	1.08	0.39	0.55	0.57	—
Brill. Cres. Blue	—	3.22	—	—	—	—	0.13	—	—	—
Brill. Green	—	3.45	4.8	4.8	6.25	—	3.22	4.8	5.0	6.25
Chrysoidin Y (not certified)	0.86	0.66	—	0.61	—	2.21	1.65	—	1.30	—
Congo Red	—	5.6	—	—	—	0.19	0.033	0.022	0.027	—
Crystal Violet	1.68	0.22	0.62	0.94	1.05	13.87	9.8	10.00	8.35	10.00
Eosin Y (Na salt)	44.2	33.3	37.00	37.00	28.00	2.18	2.44	1.95	3.03	1.85
Ethyl eosin	0.03	0.053	—	—	—	1.13	1.21	—	—	—
Fuchsin, acid	—	12.00	12.5	—	—	—	0.3	0.15	—	—
Fuchsin basic (Pararos. chl.)	0.26	2.40			0.31	5.93	9.1			3.70
Fuchsin basic (Pararos. acet.)	4.15					13.63				
Fuchsin basic (Rosan. chl.)	0.39		0.59	0.37		8.16		6.25	6.25	
Hematoxylin		1.0	1.2	—	—	—	30.00	39.00	—	—
Methylene blue (chl.)	3.55	3.03	4.5	4.35	3.21	1.48	1.70	1.88	1.82	1.75
Methyl green	—	4.8	4.8	—	—	—	0.75	0.38	—	—
Neutral red	5.64	2.70	2.85	—	—	2.45	2.40	2.18	—	—
Orange G	10.86	15.5	18.00	17.00	—	0.22	0.19	0.195	0.22	—
Pyronin Y	8.96	6.25	7.7	—	—	0.60	0.67	0.43	—	—
Rosolic acid (not certified)	—	0.15	—	0.067	—	—	26.00	—	33.00	—
Rose bengal	36.25	23.00	—	—	—	7.53	4.8	—	—	—
Safranin O	5.45	4.35	2.95	2.17	—	3.41	2.56	2.70	2.13	—
Sudan III	0	0	?	?	—	0.15	0.095	0.095	0.12	—
Toluidine blue O	3.82	5.9	3.4	—	—	0.57	0.41	0.34	—	—
Wright stain	—	0.091	—	—	—	—	0.23	—	—	—

*Data in this column taken from Table 6.

†The letters A, B, C, and D refer to American stain companies.

APPENDIX II
METHODS FOR TESTING BIOLOGICAL STAINS

In the examination of stains submitted to the Biological Stain Commission for certification, procedures for testing them have been worked out for the most common stains and were originally published by Pederson, Conn and Melin (1933–4). They may be regarded as assay methods used in evaluating dyes as biological stains.

The original methods included determinations of light absorption by means of a visual spectrophotometer; using that instrument, it proved most practical to express the color characteristics of a sample by a simple ratio of color densities at two selected wavelengths. Later, the availability of a photoelectric spectrophotemeter made it possible to characterize stains by complete absorption spectra and by exact location of the absorption maximum. The same type of ratio has been retained, however, as additional characterization.

It has proved possible to assay (as to actual dye content) by spectrophotometry those stains whose manufacture is apparently sufficiently standardized to result in quite identical spectral characteristics. The color density at the maximum absorption of such stains is easily related to the dye content as determined by other methods. In the case of stains in which various samples show considerable variation in the position of the absorption peak, it is impossible to establish the spectrometric assay, and it has proved necessary to re-evaluate the titanous chloride method, in which some changes have been made.

The following pages give the methods in use at the time the present edition of this book is going to press. See Stotz *et al.* (1950), from which much of the following is quoted.

GENERAL

Spectral Characteristics

A 50 mg. sample of the homogenous (powdered if necessary) stain is weighed to the nearest 0.5 mg. on a Roller-Smith torsion balance. The sample is transferred quantitatively to a 250 ml. volumetric flask and approximately 225 ml. of the appropriate solvent added. The flask is then vibrated on a Boerner shaker[1] for 20 minutes to insure complete solution of the dye. The solution is made up to the mark with solvent, thoroughly mixed, and an appropriate aliquot taken for further dilution to provide a satisfactory dye concentration for spectrophotometric work.

Maximum accuracy of weighing and insured complete solution of the dye is of course dispensable if the spectrophotometric assay is not employed.

Matched Corex cells of 1 cm. light path are employed in the Beckman DU spectrophotometer, the reference cell containing the same solvent as the dye solution. Spectrophotometric readings are taken at least every 5 mμ and usually at smaller intervals in the area of the absorption maximum. An actual reading at the exact peak is of course desirable for spectrophotometric assay.

The older method of expressing the shape of the curve by the ratio of color densities at two predetermined wavelengths loses its significance if the exact peak is known, since any differences in the position of the peak necessarily cause a change

[1]Arthur H. Thomas Co.

in the ratio. More elaborate methods have been used to express the shape of the curve independently of the peak position (Lillie and Roe, 1942) but we have chosen the simple ratio of the color densities at minus and plus 15 mμ of the peak wavelength found with the given sample. These densities can be estimated from the plotted spectrum or an absolute figure actually measured after location of the peak.

Titanous Chloride Assay

A study of the titanous chloride standardization, use of the solution, and the dye titration itself led to various modifications. Titanous chloride solutions are standardized against ceric sulfate, which in turn is standardized against arsenious oxide. Both titrations employ ferrous-o-phenanthroline indicator which gives sharp endpoints.

Sodium Arsenite—Dissolve 4.947 g. of U. S. Bureau of Standards arsenious oxide (As_2O_3) in a 600 ml. beaker containing 200 ml. of distilled water and 5 g. of sodium hydroxide pellets. After complete solution add 13 ml. of concentrated hydrochloric acid, mix, and then add 10 g. of sodium bicarbonate slowly to prevent loss by spray. Transfer the solution quantitatively to a 1-liter volumetric flask, dilute to the mark, and mix. The resulting solution is 0.1*N*.

Ceric Sulfate—Weigh 66.5 g. of anhydrous ceric sulfate [$Ce(SO_4)_2$] into a 600 ml. beaker and add 28 ml. of concentrated sulfuric acid. Add cautiously about 30 ml. of distilled water with stirring. Heat the solution, stir, and add successive portions of water until the salt is completely dissolved. Cool, transfer the solution to a 1-liter volumetric flask and dilute to the mark. This approximately 0.1 *N* ceric sulfate solution is completely stable.

Iodine Monochloride Catalyst—Dissolve 10 g. of potassium iodide (KI) and 6.74 g. of potassium iodate (KIO_3) in 90 ml. of distilled water. Add 90 ml. of concentrated hydrochloric acid and mix. This solution is 0.5 molar in iodine monochloride. It should be stored in the dark.

Standardization of Ceric Sulfate—Pipette exactly 25.0 ml. of 0.1 *N* sodium arsenite into a 200 ml. Erlenmeyer flask, add 20 ml. of concentrated hydrochloric acid, and 2.5 ml. of 0.005 *M* iodine chloride (dilute above stock). Dilute the mixture to about 100 ml., add 1 drop of ferrous-o-phenanthroline indicator[2], and titrate with the ceric sulfate until the orange color of the indicator returns only slowly after dropwise addition of the ceric solution. At this stage warm the solution to 50° C., add another drop of indicator, and continue the titration dropwise until a single drop produces a light green solution with no return of the orange color for at least one minute. The endpoint is sharp and reproducible. The normality of the ceric sulfate solution is calculated as follows:

$$\text{N ceric} = \frac{25 \times 0.1}{\text{ml. ceric}}$$

Titanous Chloride Solution—The stock solution is 20% titanous chloride stabilized with hydrochloric acid[3]. An approximately 0.05 *N* solution is prepared by diluting 8.0–8.5 ml. of the stock solution to 200 ml. with distilled water. Storage

[2]"Ortho-Phenanthroline Ferrous Complex (Ferroin) 0.025 *M*". Purchased from the G. Frederick Smith Chemical Co., Columbus, Ohio.

[3]LaMotte Chemical Products Co., Baltimore, Md.

experiments with this diluted titanium solution showed that it could be stored under mineral oil for a normal working day without change, and for 20 hours with approximately a 1–2% decrease in strength. In practice the solution was placed in a burette equipped with a side reservoir, and both surfaces of the solution were covered with ½ inch of mineral oil. A fresh solution was made daily and the solution standardized against ceric sulfate as follows: Exactly 5 ml. of standard ceric sulfate was placed in a 50 ml. Erlenmeyer flask, and 5 ml. of concentrated sulfuric acid added. After adding a drop of ferrous-o-phenanthroline indicator, the solution was titrated with the stored titanium solution. The initial yellow color of the solution passes through a green stage, then nearly colorless, and finally a single drop of Ti^{+++} produces the orange color of the reduced indicator. The titration is rapid and the endpoint exceedingly sharp.

Dye Titration with Titanous Chloride

Minor changes have been made in the dye titration procedure. Smaller amounts of dye are used and a more dilute titanous chloride solution is employed than has been recommended in "Biological Stains, 5th Ed." In the case of most dyes we have preferred a strong acetate buffer to maintain a more constant pH during the titration, although in some cases the tartrate or bitartrate buffer has been retained. We also prefer to use nitrogen gas washed through two titanium-chloride-safranin bottles to maintain more anaerobic conditions and minimize autoxidation of reduced dyes during the titration. Following is a general description of the titanous chloride assay as carried out in our laboratory. Specific variations will be noted under the individual dyes.

The titration is carried out in an ordinary "Mason" jar of about 350 ml. capacity and a 2½ inch diameter mouth. A tight-fitting rubber stopper contains five holes for the accommodation of a gas inlet, gas outlet, the burette extension tip, a thermometer and a ground glass sleeve for the shaft of a stirring propeller. The titration jar is mounted on an asbestos covered hot-plate. Water-pumped tank nitrogen is led through two successive gas-washing bottles[4] containing titanous chloride safranin. The latter solution is made by diluting 40 ml. of 20% titanous chloride and 40 ml. of concentrated hydrochloric acid to 500 ml. with distilled water. After filling the gas-washing bottles, approximately 10 mg. of safranin is added to each bottle. This mixture is replaced by fresh solution every two or three days depending on amount of use.

The weighed dye sample is dissolved in the appropriate solvent in the titration jar, and buffer added. The total volume of dye mixture is usually 200 ml. The solution is stirred, heated if necessary, and the nitrogen passed through the jar for at least fifteen minutes before the titration is started. Like most titrations, the first run locates the approximate endpoint so that a second trial can be rapid and the type of endpoint color change familiar.

The per cent dye is calculated by the general formula:

$$\text{per cent dye} = \frac{\text{ml. } TiCl_3 \text{ used} \times \text{normality of } TiCl_3 \times \text{mol. wt. of dye} \times 100}{\text{Wt. of sample} \times \text{No. of hydrogen equivalents} \times 1000}$$

General Approach to Examination of Individual Stains—Study of the individual

[4]Fisher-Milligan Gas Washer Bottles.

stains by the general methods described consisted of measuring the spectra of a series of samples of recent origin and of satisfactory staining quality. Ratios of the color density at the peak (D–peak) to previously recorded assay values were then calculated and compared. If these ratios (Assay/D–peak) were reasonably constant, the chemical assays were carefully repeated, the ratios recalculated, and the average value used to serve as the necessary factor for spectrophotometric assay of the stain. If the ratios proved not to be reasonably constant, either the spectrophotometric data or the chemical assay was considered to be at fault. Spectrophotometric assay was obviously unsuitable when representative samples of the stain showed great variation in the position of the peak or other spectral characteristics. In such cases the chemical assay proved to be the method of choice.

In a few cases the titanous chloride assay was not entirely satisfactory, and it was difficult to establish a figure for per cent purity. In such cases nitrogen analyses were made and the per cent dye calculated on this basis. This figure in turn served to standardize the spectrophotometric assay of the dye.

It should be noted that the methods here reported are designed to provide a quick chemical and spectrophotometric evaluation of commercial stains as they are received for certification. As such, the standards set are based primarily on past commercial samples and their satisfactory use in staining procedure. From the strictly chemical standpoint it is recognized that "per cent dye" as determined by titanous chloride, spectrophotometry, or color base precipitation cannot adequately describe the true content of the dye specifically named.

CERTAIN NITRO AND AZO DYES

Qualitative and quantitative determinations of the dyes in this group and of the ones following are, for the most part, the same as the general methods outlined at the beginning of the chapter. Where special treatment is required it will be found under the individual dye.

Martius Yellow, C. I. No. 9

Identification: Martius yellow is the monosodium or calcium salt of 2:4-dinitro-1-naphthol. $C_{10}H_5N_2O_5Na$ or $(C_{10}H_5N_2O_5)_2Ca$. It is identified by the following method: Dissolve 50 mg. of martius yellow in 250 ml. of distilled water. Dilute 10 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 431–440 mμ, ratio P–15/P+15 is from 1.00 to 1.12.

A marked distinction is observed in the solubilities of the two salts in water, the sodium salt being soluble to the extent of 4.5 parts in 100 parts of water at 26° C., whereas only 0.05 parts of the calcium salt are soluble in 100 parts of water at the same temperature.

Method of Analysis: Dissolve 100 mg. of dye in 200 ml. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling, and titrate with 0.05 N $TiCl_3$. A change from orange to a straw yellow indicates the endpoint. The following data are used in calculating the percentage of anhydrous dye in the original sample:

(1) Molecular weight, $C_{10}H_5N_2O_5Na$	256.153
Hydrogen equivalents per mol of dye	12.
ml. of N/10 $TiCl_3$ per gram of dye	468.467

or (2)	Molecular weight, $(C_{10}H_5N_2O_5)_2Ca$	506.392
	Hydrogen equivalents per mol of dye	24.
	ml. of N/10 $TiCl_3$ per gram of dye	473.944

Biological tests: Martius yellow is tested as a counterstain to resorcin blue for staining pollen tubes in the style. Slender styles and ovaries, while still moist, are crushed between two slides; while larger ones are treated similarly after sectioning longitudinally by hand. The material is either stained on the slide or immersed in the stain in pieces in a small dish, for 2 to 5 minutes. The staining solution consists of 5 mg. resorcin blue and 5 mg. martius yellow in 10 to 15 ml. water, with a few drops of 1% aqueous ammonia added to bring the reaction to about pH 8, as shown by the solution assuming an olive color. The material is mounted in the stain or else in water of the same reaction, and examined with a powerful light. A good sample is one with which the pollen tubes show blue on a light yellowish green background.

Orange G, C. I. No. 27

Identification: Orange G is the disodium salt of benzene-azo-2-naphthol-6:8-disulfonic acid, $C_{16}H_{10}N_2O_7S_2Na_2$. It is identified by the following method: Dissolve 50 mg. of orange G in 250 ml. of distilled water. Dilute 15 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 476–481 mμ; ratio P–15/P+15 is from 0.88 to 0.94.

Method of Analysis: Dissolve 10 mg. of dye in 175 ml. of distilled water, add 25 ml. of acetate buffer pH 4.0, heat to boiling, and titrate with 0.05 *N* $TiCl_3$. The end point is a sharp change from brownish-yellow to a pale yellowish-green. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	452.370
Hydrogen equivalents per mol of dye	4.
ml. of N/10 $TiCl_3$ per gram of dye	88.424

For certification, samples of this stain must contain not less than 80% anhydrous dye.

Biological Tests: Orange G is tested (1) in Mallory's connective tissue stain and (2) as a counterstain in histology and (3) in cytology.

It is tested in Mallory's connective tissue stain on animal tissue fixed in Zenker's solution and embedded in paraffin. The sections are stained 1–5 minutes in a 0.5% aqueous solution of acid fuchsin and transferred directly to a solution containing 0.5 g. anilin blue W.S., 2 g. orange G, and 100 ml. of a 1% aqueous solution of phosphotungstic acid, for 10 to 20 minutes or longer; dehydrated in several changes of 95% alcohol, then absolute alcohol; cleared in xylene and mounted in xylene, colophonium or balsam. With a good sample, the red blood corpuscles and myelin sheaths are yellow and elastic fibers pale pink or yellow.

For its histological use it is tested on animal tissue fixed in Bouin's or Zenker's solution and embedded in paraffin. A 0.5% solution in 95% alcohol is employed and applied for 15 seconds or more as a counterstain after Heidenhain's hematoxylin. (See Eosin Y).

For cytological work, root tip material, fixed in Navashin's or Flemming's fluid,

is stained in a 1% aqueous crystal violet for 5 min., rinsed in distilled water and transferred to equal parts of a 1% iodine and 1% KI in 80% alcohol for 15 to 50 seconds. The sections are next dehydrated in absolute alcohol for 4 to 5 seconds and differentiated in a saturated solution of orange G in clove oil. They are then cleared in xylene and mounted. In both of these procedures a stain is required which gives a good contrast to the nuclear dye.

Janus Green B,* C. I. No. 133

Identification: Janus green B is diethylsafranin-azo-dimethylanilin, $C_{30}H_{31}N_6Cl$. It is identified by the following method: Dissolve 50 mg. in 125 ml. of 95% alcohol and then dilute to 250 ml. with distilled water. Take 10 ml. of this solution and dilute to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 610–623 mμ; ratio P–15/P+15 is from 0.98 to 1.02.

Method of analysis: Dissolve 100 mg. of dye in 50 ml. of 95% alcohol, add 150 ml. of distilled water and 10 g. of sodium acid tartrate, heat to boiling, and titrate with 0.05 *N* $TiCl_3$.

The dye solution undergoes several color changes on reduction, but the final end point, usually a clear reddish-brown, is easy to recognize. The following data are used to calculate the percentage of anhydrous dye in the original sample:

Molecular weight	511.053
Hydrogen equivalents per mol of dye	6.
ml. of N/10 $TiCl_3$ per gram of dye	117.405

Samples of this stain should contain not less than 50% anhydrous dye.

Biological Tests: Janus green is tested, mixed with neutral red, for the supravital staining of blood. Saturated stock solutions of the two dyes are prepared by adding 125 mg. of neutral red to 50 ml. of neutral absolute alcohol and 125 mg. of Janus green to 62.5 ml. of neutral absolute alcohol. Both of these solutions are stable. A dilute stock solution of neutral red is prepared by adding 1.1 ml. of the saturated neutral red to 10 ml. of neutral absolute alcohol. This solution is also stable. In testing Janus green, 0.4 ml. of the saturated solution of this dye is added to 3 ml. of the dilute solution of neutral red. This mixture is not stable and must be used promptly.

Slides are washed and dried carefully, after treatment in bichromate cleaning fluid, and are then flooded with the mixed stain, which is allowed to drain off. The slides are then dried quickly in front of a Bunsen flame. A drop of fresh blood is placed on a cover slip which is inverted on top of the slide having the film of dried stain. The edges of the cover slip must be sealed with vaseline of high melting point.

By this technic, with good samples of both dyes, the basophilic granules become brilliant scarlet, the eosinophilic granules yellow or light orange, while the neutrophilic granules are salmon color. It is essential that no staining of the nuclei should be obtained which, if it occurs, indicates that the cells are killed. All the

*The formula given in the Colour Index for Janus green B has been found incorrect for all samples furnished in this country. The dye usually encountered as a biological stain has the formula given here. However, German samples of the dye, although similar, are not identical with the usual stain sample.

areas that stain with neutral red take up the dye quickly, except in the case of monocytes, which require about 10 minutes. The mitochondria show up after about 15 minutes as small blue dots or rods. The cells should live in good condition for at least an hour.

Methyl Orange, C. I. No. 142

Identification: Methyl orange is the sodium salt of p-sulfobenzene-azo-dimethylanilin, $C_{14}H_{14}N_3O_3SNa$. It is identified by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 5 ml. of this solution and 1 ml. of 1*N* HCl to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 506–508 mμ, ratio P–15/P+15 is from 0.95 to 0.96.

Method of analysis: Dissolve 100 mg. of dye in 200 ml. of distilled water, add 15 g. sodium acid tartrate, heat to boiling, and titrate with 0.05 *N* $TiCl_3$. The end point is sharp, giving a colorless solution. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	327.333
Hydrogen equivalents per mol of dye	4.
ml. of N/10 $TiCl_3$ required per gram of dye	122.200

Samples of this stain should contain not less than 85% anhydrous dye.

Biological Tests: Methyl orange is tested in saturated alcoholic solution as a counterstain, applied for 15 minutes following crystal violet, or 1 or 2 minutes following Harris' or Heidenhain's hematoxylin, on paraffin sections of animal tissue fixed in Bouin's or of plant tissue fixed in Flemming's fluid. A good sample is one which shows a good contrast to the nuclear stain.

Orange II, C. I. No. 151

Identification: Orange II is the sodium salt of p-sulfobenzene-azo-2-naphthol, $C_{16}H_{11}N_2O_4SNa$. It is identified by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 10 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 483–487 mμ; ratio P–15/P+15 is 0.96

Method of analysis: Dissolve 100 mg. of dye in 200 ml. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling and titrate with 0.05 *N* $TiCl_3$. Reduce to a practically colorless solution. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	350.321
Hydrogen equivalents per mol of dye	4.
ml. of N/10 $TiCl_3$ per gram of dye	114.179

Samples of this stain should contain not less than 85% anhydrous dye.

Biological Tests: Orange II is tested by the same procedure as in the case of methyl orange.

Sudan III, C. I. No. 248

Identification: Sudan III is benzene-azo-benzene-azo-2-naphthol, $C_{22}H_{16}N_4O$. It is identified by the following method: Dissolve 50 mg. in 250 ml. of benzene. Dilute 2 ml. of this solution to 50 ml. with benzene. Read in Beckman spectrophotometer. Absorption maximum 508–511 mμ; ratio P–15/P+15 is from 0.97 to 1.01.

Method of Analysis: The low solubility of this dye interferes with its determination, and it is therefore sulfonated before titration. Weigh about 1 g. of dye into a 125 ml. beaker-flask, add 25 ml. of 23% fuming sulfuric acid, and heat on the steam bath for about one hour. Allow the mixture to cool and pour into 500 ml. of distilled water in a one-liter volumetric flask. After cooling to room temperature, the solution is brought up to the mark.* Pipette a 100 ml. aliquot of the sulfonated solution into the titration flask, add 100 ml. of distilled water and 30 g. of sodium acid tartrate, heat to boiling, and titrate with 0.05*N* $TiCl_3$. The color change near the end point is very gradual, going from orange to a lemon-yellow. The disappearance, however, of the last trace of orange is easily detected. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	352.380
Hydrogen equivalents per mol of dye	8.
ml. of N/10 $TiCl_3$ per gram of dye	227.026

Samples of this stain should contain not less than 75% anhydrous dye.

Biological Tests: Sudan III is tested as a fat stain. The method employed is the same as that given under the following dye. Sudan III, however, is not expected to give as intense a coloration as Sudan IV.

Sudan IV, C. I. No. 258

Identification: Sudan IV is o-toluene-azo-o-toluene-azo-2-naphthol, $C_{24}H_{20}N_4O$. It is identified as follows: Dilute 1 ml. of sulfonated solution of Sudan IV to 200 ml. with distilled water. Sulfonating procedure is outlined under Sudan III. Read in Beckman spectrophotometer. Absorption maximum 522–529 mμ; ratio P–15/P+15 is from 0.94 to 0.99.

Method of Analysis: The low solubility of this dye interferes with its determination, and it is therefore sulfonated before titration, by the same procedure as that outlined for Sudan III. The percentage of dye in the original sample is calculated from the following data:

Molecular weight	380.432
Hydrogen equivalents per mol of dye	8.
ml. of N/10 $TiCl_3$ per gram of dye	210.286

For certification, samples must contain not less than 80% anhydrous dye.

Biological Tests: Sudan IV is tested as a fat stain. Frozen sections of formalin-fixed tissue containing fat, or portions of the mesentery of some animal, serve for material. Sections are dipped for an instant in 70% alcohol, stained 2-5 minutes in

*The red solution obtained on the dilution of the blue sulfonation product should be clear. If the sulfonation has been incomplete, the solution is cloudy.

Sudan IV solution (Sudan IV, 0.1 g. in a mixture of 50 ml. 70% alcohol and 50 ml. acetone C.P.). They are washed quickly in 70% alcohol and transferred to distilled water, and counterstained in alum hematoxylin, washed thoroughly in tap water and mounted in glycerin or glycerin jelly. A good sample should give a sharp reddish-orange color to the fat globules.

Bismarck Brown Y, C. I. No. 331

Identification: Bismarck brown Y is the dihydrochloride of benzene-m-disazo-bis-m-phenylenediamine, $C_{18}H_{20}N_8Cl_2$. It is identified as follows: Dissolve 50 mg. in 125 ml. of 95% alcohol, then dilute to 250 ml. with distilled water. Dilute 10 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 461–475 mμ; ratio P–15/P+15 is 1.01.

Method of Analysis: Dissolve 100 mg. of the dye to be tested in 100 ml. of 95% alcohol and 100 ml. of distilled water, add about 10 g. of sodium acid tartrate, heat to boiling, and titrate with 0.05*N* $TiCl_3$. The end point, which ranges from pale yellow to brownish-yellow, is not always sharp, and is best observed by adding the titanous chloride solution 4 drops at a time. The following data are used for calculating the percentage of anhydrous dye in the original sample:

Molecular weight	419.318
Hydrogen equivalents per mol of dye	8.
ml. of N/10 $TiCl_3$ per gram of dye	190.787

For certification, samples of this stain must not contain less than 45% anhydrous dye.

Biological Tests: Bismarck brown Y is tested in a 1% aqueous solution for staining mucous in goblet cells of the intestine, or cartilage in the trachea or in embryonic material, fixed in one of the usual fixatives and embedded in paraffin. The sections are stained for about 5 to 10 minutes, rinsed in 95% alcohol, and transferred to the 0.5% aqueous methyl green solution until they appear dark green, after which they are dehydrated, cleared and mounted. A good Bismarck brown should show light brown mucous and deep brown cartilage.

It is also tested in Neisser's method for diphtheria. Smears are prepared in the usual manner and fixed with gentle heat. They are stained 2 or 3 seconds in acetic methylene blue (methylene blue, 0.1 g.; 95% alcohol, 2 ml.; glacial acetic acid, 5 ml.; distilled water, 95 ml.). They are then washed in tap water; stained 3 to 5 seconds in 0.2% Bismarck brown dissolved in boiling water, which has been filtered; washed, dried and unmounted. A good stain will show the bacilli uniformly brown or may show at one or both ends, dark blue round body. True diphtheria organisms usually show blue bodies, pseudotypes few.

Congo Red, C. I. No. 370

Identification: Congo red is the disodium salt of diphenyl-disazo-bis-l-naphthylamine-4-sulfonic acid, $C_{32}H_{22}N_6O_6S_2Na_2$. It is identified as follows: Dissolve 50 mg. of Congo red in 250 ml. of distilled water. Dilute 10 ml. of this solution and 2 ml. 1% Na_2CO_3 to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 497–499 mμ; ratio P–15/P+15 is from 1.00 to 1.03.

Method of Analysis: Dissolve 0.1 g. of the dye in 125 ml. of distilled water, add 30 ml. of 30% sodium tartrate solution, heat to boiling, and titrate with N/10 $TiCl_3$. Titrate rapidly until near the end point, then slowly. The end point is sharp, a practically colorless solution resulting. Data for the calculation of the percentage of anhydrous dye in the sample are as follows:

Molecular weight	696.658
Hydrogen equivalents per mol of dye	8.
ml. of N/10 $TiCl_3$ per gram of dye	114.834

For certification, samples of this stain must contain not less than 75% anyhdrous dye.

Biological Tests: Congo red is tested on paraffin sections of animal tissue fixed in Bouin's fluid. The sections are stained 5 minutes in Mayer's hemalum, dipped in tap water once or twice, and transferred directly into 0.5% aqueous solution of Congo red and left therein for 1 minute. They are then rinsed in tap water, run up through the alcohols, cleared and mounted. The criteria by which the sample is judged are as follows: Congo red should give a bright cytoplasmic stain with a certain amount of differentiation from orange to reddish. Erythrocytes, for instance, should be light orange and the spindle fibers in mitotic figures should be deep orange to light red.

Chlorazol Black E, C. I. No. 581

Identification: Chlorazol black E has the empirical formula $C_{34}H_{25}N_9O_7S_2Na_2$. It is identified as follows: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 5 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 598–602 mμ; ratio P–15/P+15 is from 0.97 to 1.00.

Method of Analysis: No method of analysis has been devised for chlorazol black E.

Biological Tests: Chlorazol black E is tested on paraffin sections of animal tissue fixed in Zenker's fluid, and on plant tissue fixed in Flemming's or Bouin's. The sections are stained 5-10 minutes in a 1% solution in 70% ethyl alcohol. The excess dye is drained off and the sections are dehydrated, cleared and mounted in balsam. No mordant and no differentiation are necessary. The tissue elements should stain varying shades of green, gray and black, and sharp differentiation should be evident. Too general appearance of green is regarded as undesirable.

In using this as an auxiliary stain in making chromosome counts on plants, the above staining solution is applied 5-25 minutes to the dissected tissue, fixed in acetic acid (1 volume to 3 volumes of alcohol), rinsed in three changes of 70% alcohol and the material is then transferred to a slide. A drop of aceto-carmine (boiling 45% acetic acid saturated with carmine and filtered) is added and then covered with a cover glass, heated, flattened and sealed. The chromosomes should stain deep reddish black with a fairly clear cytoplasm.

Alizarin Red S, C. I. No. 1034

Identification: Alizarin red S has the empirical formula $C_{14}H_7O_7S$ Na. It is identified as follows: Dissolve 50 mg. in 250 ml. of 0.1 *N* NaOH. Dilute 15 ml. of this solution to 200 ml. with 0.1 *N* NaOH. Read in Beckman spectrophotometer.

This dye has two absorption maxima, one at 555–558 mμ, the other at 594–599 mμ, ratio P–15/P+15 for first maximum is 1.02.

Method of Analysis: No method of analysis for alizarin red S is known.

Biological Tests: Alizarin red S is tested on small vertebrates, fixed 2–4 days or longer in 95% alcohol, by the following technic: Place specimen in 1% aqueous KOH until the bones are clearly visible through the surrounding tissues, transfer to dilute (0.0025–0.01%) alizarin red S in 1% aqueous KOH and allow to stand until desired degree of staining is obtained; complete the clearing by placing in a mixture of 1 vol. glycerin to 4 vol. of 1.25% aqueous KOH (glycerin 20 ml., KOH 1 g., water 79 ml.) and continue through increasing concentrations of glycerin. Store in glycerin alone. The bones should be red, soft tissue transparent and unstained.

Sudan Black B

Identification: The empirical formula for Sudan black B is $C_{24}H_{22}N_6$. The method of identification is as follows: Dissolve 50 mg. in 250 ml. of 95% alcohol. Dilute 10 ml. of this solution to 200 ml. with 95% alcohol. Read in Beckman spectrophotometer. Absorption maximum 596–605 mμ; ratio P–15/P+15 is from 0.99 to 1.02.

Method of Analysis: A method for determinating dye content has not yet been perfected.

Biological Tests: Sudan black B is tested for fatty material in bacteria in Burdon's technic. Culture of *B. cereus* is grown at room temperature for 18–24 hr. on potato slant or glucose starch agar. Prepare film and air dry. Flood entire slide with Sudan black B solution (Sudan black B 0.3 g., 70% alcohol, 100 ml.). After bulk of dye has been dissolved, shake thoroughly at intervals during the day and allow to stand over night. Flood slide with the stain and allow it to remain undisturbed 5–15 min., drain off excess stain, blot dry, clear with xylene, blot dry. A satisfactory sample should show intercellular fat dark blue, cytoplasmic membrane intensely stained, dark blue black.

THE QUINONE-IMINE DYES

Thionin (Lauth's Violet), C. I. No. 920

Identification: Thionin is diaminodiphenazthionium chloride, $C_{12}H_{10}N_3SCl$. It is identified by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 5 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 598–599 mμ; ratio P–15/P+15 is from 1.15 to 1.35.

Method of Analysis: The determination of dye content is made by the spectrophotometric method, calculating by the formula: Percent dye = D-peak × 97. D-peak (color density at peak) is measured on dye solution as described under "Identification".

Samples of this stain should contain not less than 85% anhydrous dye.

Biological Tests: Thionin is tested as a stain for frozen sections, also in Stoughton's method for sections of plant pathological material. For frozen sections the dye is prepared in a 0.5% solution in 20% ethyl alcohol. The sections, immediately after cutting, are immersed in water and placed briefly (1 to 2 minutes) in the stain, then washed and mounted in water for examination. Good differentiation of the nuclei is regarded as the criterion of á satisfactory sample. In the Stoughton method, plant pathologic material is fixed in one of the usual botanical fixatives, paraffin sections are stained for 1 hour in 0.1% thionin in 5% aqueous phenol, dehydrated in successively stronger alcohols and differentiated in a saturated solution of orange G in absolute alcohol for 1 minute. They are then washed in absolute alcohol, cleared in xylene and mounted in balsam. Fungal parasites should be violet to purple; cell walls, yellowish or green; lignified tissue, blue; host nuclei, blue with purple nucleoli; chromosomes, deep blue; and nuclei of the fungal hyphae or spores, deep purple.

Methylene Blue, C. I. No. 922

Identification: Methylene blue is tetramethyldiaminodiphenazthionium chloride, (tetramethyl thionin) $C_{16}H_{18}N_3SCl$. This dye is sometimes marketed for textile purposes as the zinc chloride double salt, but the dye in this form is not recommended for use as a biological stain. It is identified as follows: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 3 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 665 mμ; ratio P–15/P+15 from 1.37 to 1.70.

Method of analysis: The dye content is determined spectrophotmetrically calculating from the formula: Percent dye = D-peak × 127. D-peak (color density at peak) is measured on dye solution as described under "Identification".

For certification, samples of this stain must contain not less than 82% anhydrous dye.

Biological Tests: Methylene blue is tested for histological and bacteriological staining and as a constituent of Wright's blood stain. As an histological stain it is tested on paraffin sections of tissue fixed in Zenker's fluid, with phloxine as a counterstain, by the procedure given on page IA_3-20 of Staining Procedures. A good sample should show good nuclear staining without removing the phloxine from the cytoplasm.

As a bacteriological stain it is tested for staining the diphtheria organism and for staining bacteria in milk. For the former purpose smears from a throat culture of a case of diphtheria are stained in three different solutions of the sample under examination; namely Loeffler's formula (methylene blue, 0.3 g.; 95% ethyl alcohol, 30 ml.; 0.01% KOH, 100 ml.), alcoholic aqueous methylene blue (same, but with distilled water in place of 0.01% KOH), and also in a 1% aqueous solution of the dye. The solutions are applied to the preparation for a few seconds which is then washed in tap water and examined under the microscope to see if the typical barred or granular structure shows. A good sample should show this typical staining with all three solutions.

For staining bacteria in milk, 0.01 ml. is placed on a microscopic slide and smeared over an area of 1 sq. cm. with a stiff needle, dried with gentle heat on a level surface,

dipped in xylene a few minutes to remove the fat, immersed in 90–95% ethyl alcohol a few minutes to fix the smear to the slide. It is then dipped 2–4 times in methylene blue solution (methylene blue, 0.3 g.; 95% ethyl alcohol, 30 ml., after dissolving, mixed with 100 ml. distilled water), washed briefly in 90–95% ethyl alcohol until the intense blue color changes to a faint tinge. (This decolorizing may be omitted if the staining period has been made briefer, 10-15 seconds, or if a more dilute staining fluid has been employed). The slide is then dried and examined. A good sample should not remove the milk smear from the slide and should show deeply stained bacteria in a light blue background.

In testing as a constituent of Wright's stain, the same procedure is followed as in the case of eosin.

Methylene Azure, C. I. No. 923

(azure a and azure b)

Identification: Methylene azure is obtained by the oxidation of methylene blue, which results in a mixture of di-, tri- and tetramethyldiaminodiphenazthionium chlorides in varying proportions. The first-mentioned product, asymmetrical dimethyl thionin or azure A is $C_{14}H_{14}N_3SCl$; the second, Azure B or trimethyl thionin, is $C_{15}H_{16}N_3SCl$. They are identified by the following method: Dissolve 50 mg. sample in 250 ml. of distilled water. Dilute 3 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum of azure A is 622.5–632.5 mμ, of azure B, 647.5–654 mμ; the ratio P–15/P+15 for azure A is from 0.94 to 1.05, for azure B is from 0.94 to 1.12.

Method of Analysis: Dissolve 100 mg. of dye in 175 ml. of distilled water, add 25 ml. of pH 4.0 acetate buffer (6 volumes of 50% $NaAc{\cdot}3H_2O$ + 4 volumes of glacial acetic acid), heat to boiling, and titrate with 0.05 *N* $TiCl_3$ to a yellow endpoint.

If qualitative examination has shown the dye sample to be almost pure azure A or almost pure azure B, the percentage of anhydrous dye in the original sample may be determined from the following data. If the dye is found to be a mixture, the actual dye content cannot be accurately determined.

	Azure A	*Azure B*
Molecular weight	291.793	305.819
Hydrogen equivalents per mol of dye	2.	2.
ml. of N/10 $TiCl_3$ per gram of dye	68.541	65.398

Samples of this stain must contain not less than 55% anhydrous dye.

Biological Tests: Azure A is tested on paraffin sections of animal tissue fixed in Bouin's or Zenker's fluid by means of Haynes' modification of French's stain. The paraffin is removed by applying each of the following for 3 minutes: xylene, absolute alcohol, 95% ethyl alcohol, and distilled water. The sections are stained for 5 minutes in 1.5% aqueous azure A, treated 5-10 seconds with absolute ethyl alcohol, counterstained with a saturated solution of ethyl eosin in clove oil for 30 seconds, then xylene, 10-30 seconds, xylene, two changes of 1-2 minutes each, and mounted in xylene balsam. Good nuclear staining is regarded as the criterion of a satisfactory sample.

It is also tested as a constituent of the tetrachrome stain made up as follows: methylene blue chloride, 1.0 g.; azure A, 0.6 g.; methylene violet, 0.2 g.; eosin Y, 1.0 g. are mixed together; 0.15-0.3 g. of the mixed dry ingredients are dissolved in 100 ml. methyl alcohol (neutral, acetone free) by heating to 50°C., shaken thoroughly and left 1-2 days at 37°C., with occasional shaking. It is then filtered, after which it is tested by the same procedure as Wright's stain, page 327. With a good azure A this compound stain should color the platelets, the lobes of the polymorphonuclear cells and the nuclei of the lymphocytes a distinct purple.

Azure C

Identification: Azure C is monomethyldiaminodiphenazthionium chloride (monomethyl thionin), $C_{13}H_{12}N_3SCl$. The method of identification ia as follows: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 3 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 607.5–612.5 mμ; ratio P–15/P+15 is from 0.98 to 1.03.

Method of analysis: Dissolve 100 mg. of dye in 175 ml. of distilled water, add 25 ml. of pH 4.0 acetate buffer (6 volumes of 50% $NaAc \cdot 3H_2O$ + 4 volumes of glacial acetic acid), heat to boiling and titrate with 0.05 *N* $TiCl_3$ to a yellow endpoint. The following data are used in calculating the percentage of anhydrous dye in the sample:

Molecular weight	277.767
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	72.003

Because of the uncertain composition of samples of this stain, in that they contain varying amounts of the higher methylated thionins and thionin, no dye content is reported.

Biological Tests: Azure C is tested in the same way as azure A (see methylene azure, p. 304.)

Methylene Violet

Identification: Dissolve 25 mg. in 125 ml. of 95% alcohol, add 10 ml. of 0.1*N* HCl and dilute to 250 ml. with distilled water. Dilute 10 ml. of this solution and 20 ml. of 0.1 *N* HCl to 200 ml. with 50% alcohol, Read in Beckman spectrophotometer. Absorption maximum 579–580 mμ; ratio P–15/P+15 is from 0.99 to 1.02.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 181. D-peak (color density at peak) measured on dye solution described under "Identification".

Biological Tests: It is tested as a constituent of the tetrachrome stain made up by the formula given on p. 245. A blood smear stained with this mixture, containing a satisfactory methylene violet, shows a pure blue in the cytoplasm of the lymphocytes and a deeper blue in the granules of this cytoplasm.

Toluidine Blue O, C. I. No. 925

Identification: Toluidine blue O is usually the zinc chloride double salt of aminodimethylaminotoluphenazthionium chloride, $C_{15}H_{16}N_3SCl + ZnCl_2$, but may also be

prepared as the chloride. It is identified as follows: Dissolve 50 mg. in 250 ml. of distilled water. Dilute to 5 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 620–632.5 mμ; ratio P–15/P+15 is from 1.01 to 1.09.

Method of Analysis: Dissolve 100 mg. of dye in 175 ml. of water, add 25 ml. of pH 4.0 acetate buffer (6 volumes of 50% $NaAc \cdot 3H_2O$ + 4 volumes glacial acetic acid), heat to boiling and titrate with 0.05 *N* $TiCl_3$ to a yellow endpoint. The following data are used in calculating the percentage of anhydrous dye in the sample:

Molecular weight	305.819
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	65.398

For certification, samples of this stain must contain not less than 50% anhydrous dye.

Biological Tests: Toluidine blue O is tested as a stain for frozen sections and as a substitute for azure A in Haynes' modification of French's stain. For the former purpose the same procedure is used as in the case of thionin, p. 303; for the latter the same as in the case of azure A, p. 304.

It is also tested as a constituent of Albert's diphtheria stain, using the following method: Smears are made and fixed with gentle heat. They are stained 5 minutes in the following solution: toluidine blue, 0.15 g.; methyl green, 0.02 g.; glacial acetic acid, 1 ml.; 95% ethyl alcohol, 2 ml.; and distilled water, 100 ml. The stain is drained off without washing, and Lugol's iodine solution is applied for 1 minute; the smears are then washed briefly in tap water, blotted with filter paper and examined. A good stain shows the granules of the diphtheria bacilli black, the bars of bacilli dark green to black, the body of the cells and other bacteria light green. It is also tested in Laybourn's modification in which malachite green is used instead of methyl green.

Brilliant Cresyl Blue, C. I. No. 877

Identification: In general, samples of this stain are aminodimethylaminomethyldiphenazonium chlorides, $C_{15}H_{16}N_3OCl$, although the dye is given in the Colour Index as the diethyl derivative. The method of identification is: Dissolve 50 mg. in 125 ml. of distilled water and then dilute to 250 ml. with 95% alcohol. Dilute 5 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 626–630 mμ; ratio P–15/P+15 is from 1.10 to 1.21.

Method of Analysis: Dissolve 100 mg. of dye in 100 ml. of 95% alcohol, add 100 ml. of distilled water and 10 g. of sodium acid tartrate, heat to boiling and titrate with 0.05*N* $TiCl_3$ to the first appearance of a lighter reddish-brown color. The following data are used in calculating the precentage of anhydrous dye in the original sample:

Molecular weight	289.759
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	69.023

For certification, samples of this stain must contain not less than 50% anhydrous dye.

Biological Tests: In testing brilliant cresyl blue, fresh blood is examined under a cover glass on which a filtered 0.3% alcoholic solution of brilliant cresyl blue has been dried or blood films are made on similar cover glasses, dried and counterstained with Wright's or similar stain. The reticulum of immature red cells should be a clear-cut blue on a very pale blue (fresh) or eosin colored (stained) background. Blood platelets should stain a pale blue and be discrete, with only a minimum of precipitate or debris.

Nile Blue Sulfate (Nile Blue A), C. I. No. 913

Identification: Nile blue A is aminodiethylaminonaphthophenazonium sulfate $(C_{20}H_{20}N_3O)_2SO_4$. The method of identification is: Dissolve 50 mg. in 125 ml. of 95% alcohol and then dilute to 250 ml. with distilled water. Dilute 3 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 635–645 mμ, ratio P–15/P+15 is from 1.02 to 1.12.

Method of Analysis: Dissolve 100 mg. of dye in 100 ml. of 95% alcohol, add 75 ml. of distilled water and 25 ml. of 30% sodium tartrate solution, heat to boiling and titrate with 0.05 *N* $TiCl_3$. It is necessary to keep the solution boiling throughout the reduction, otherwise the reaction is very slow, especially near the end point. The final color change is from reddish brown to yellow. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	732.820
Hydrogen equivalents per mol of dye	4.
ml. of N/10 $TiCl_3$ per gram of dye	54.584

Samples of this stain should contain not less than 70% anhydrous dye.

Biological Tests: Nile blue sulfate is tested as a differential fat stain by the Smith and Mair technic. The material used for this test consists of insect ova fixed and preserved in neutral formalin. Sections are cut on the freezing microtome and stained with the hydrolyzed dye prepared by the technic given in the following paragraph. After staining for about 2 hours at 37° C. they are differentiated in 2% acetic acid, washed in distilled water and mounted in glycerin or Farrant's medium. A good sample is indicated by its ability to distinguish between free fatty acids (blue) and neutralized fats (red). The staining solution is prepared as follows:

A saturated aqueous solution of Nile blue sulfate is made and 0.5% H_2SO_4 is added. It is boiled under a reflux condenser for 1-2 hours. The solution is tested by shaking up a little of it in a test-tube with xylene; if a sufficient amount of the red oxazone has been formed, the xylene will assume an intensely fluorescent red color. The H_2SO_4 may be neutralized by adding an equivalent amount of NaOH or the stain may be used in acid solution.

Cresyl Violet

Identification: The American stain, cresyl violet (National Aniline Company) is aminonaphthodimethylaminotolazoxonium chloride, $C_{19}H_{18}N_3OCl$. On the other

hand, cresyl echt violet (Grübler) is a dye of unknown constitution. The method of dentification is: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 5 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. In the case of the National Aniline product, the absorption maximum is 624–630 mμ; the ratio P–15/P+15 is from 0.99 to 1.05. Cresyl echt violet (Grübler), on the other hand has absorption maxima at 550 mμ and 585 mμ, the ratio P–15/P+15 for first maximum is 1.10.

Method of Analysis: No method has yet been worked out for the German product. In the case of the National Aniline product: Dissolve 100 mg. dye in 200 ml. of 50% alcohol, add 10 g. sodium acid tartrate, heat to boiling and titrate with 0.05 *N* $TiCl_3$ to a greenish-yellow endpoint. The following data are used in calculating the percentage of anhydrous dye in the sample:

Molecular weight	339.815
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	58.856

Samples of this stain should contain not less than 88% anhydrous dye.

Biological Tests. Cresyl violet is tested on celloidin sections of central nervous tissue fixed in 10% formalin or 95% ethyl alcohol. The sections are placed (either with or without previous removal of the celloidin by means of equal parts of absolute alcohol and ether) in 0.25% aqueous cresyl violet and heated until steaming. After washing well in distilled water, they are differentiated from one to several minutes in 95% ethyl alcohol, then for a few seconds longer in Gothard's solution, and finally through two or more changes of the 95% alcohol, controlling the results under a microscope and returning to Gothard's solution if necessary. If celloidin was not removed before staining, it is now removed with equal parts of ether and absolute ethyl alcohol. The sections are dehydrated in two changes of absolute alcohol, cleared in xylene and mounted in balsam. A satisfactory sample should show nerve cells and the nuclei of other cells. The cytoplasm of some glia cells and fibroblasts may be faintly stained. The background should be clear and colorless.

Neutral Red, C. I. No. 825

Identification: Neutral red is aminodimethylaminotoluphenazonium chloride, $C_{15}H_{17}N_4Cl$. The method of identification is: Dissolve 50 mg. in 250 ml. of 50% alcohol containing 1.25 ml. glacial acetic acid. Dilute 5 ml. of this solution to 200 ml. with 50% alcohol containing 1 ml. of glacial acetic acid. Read in Beckman spectrophotometer. Absorption maximum 539–542 mμ; ratio P–15/P+15 is from 1.00–106.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 129. D-peak (color density at peak) measured on dye solution described under "Identification".

For certification, samples of this stain must contain not less than 50% anhydrous dye.

Biological Tests: Neutral red is tested for two purposes: vital and supravital staining of protozoa; differential staining of living cells of blood and connective tissues.

In testing for the first purpose, not only color intensity but relative toxicity and the stability of aqueous solutions in sunlight are determined. *Paramecium* and ery-

throcytes of *Necturus* are utilized as test materials. With *Paramecium* aqueous solutions ranging from 1:5000 to 1:100,000 concentration are employed, and one drop of concentrated culture is added to 10 ml. of the dye solution. The rate and intensity of staining of intracellular granules, the effect on ciliary activity and elevation of the pellicle, and the distortion of the organism constitute the criteria of the suitability of the dye for vital staining. In supravital testing a 1:1250 solution in absolute ethyl alcohol is applied to a glass slide, yielding the so-called dry-dye-film. Drops of freshly drawn blood are placed on the slide, covered, and sealed with warm vaseline. Observations are made at room temperature with the preparation shielded from direct heat from the source of illumination. In the erythrocyte, definite bipolar clusters of preformed granules stain immediately. Relative toxicity is judged by the rate at which induced or secondary staining patterns (granules or reticulation) appear. Corrections for percentage of dye content and the kind of salt (chloride or iodide) are necessary.

In testing for the second purpose (staining living blood and connective tissue cells), the same procedure is used as for Janus green, see p. 297.

Safranin O, C. I. No. 841

Identification: Safranin is a mixture of diaminophenylditolazonium chloride, $C_{20}H_{19}N_4Cl$ and diamino-o-tolylditolazonium chloride, $C_{21}H_{21}N_4Cl$. The method of identification is: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 3 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 530–533 mμ; ratio P–15/P+15 is from 1.14 to 1.32.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 231. D-peak (color density at peak) measured on dye solution described under "Identification".

For certification, samples of this stain must contain not less than 80% anhydrous dye.

Biological Tests: Safranin is tested as a chromosome stain in the Flemming triple stain and in combination with light green SF yellowish or fast green FCF, also the Gram stain. The procedures for the Flemming stain are the same as described below under crystal violet (p. 313). The procedure with fast green or light green as a stain is given on page 313,

Nigrosin, Water Soluble, C. I. No. 865

Identification: Nigrosin water soluble is the sodium salt of the product resulting from the sulfonation of spirit soluble nigrosin which is obtained by the interaction of anilin, anilin hydrochloride and nitrophenol (or nitrobenzene and iron). The method of identification is: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 25 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 575 mμ; ratio P–15/P+15 is from 0.94 to 0.97.

Method of Analysis: Since this dye is a variable mixture of complex compounds of unknown constitution, quantitative determination of the dye content is impossible.

Biological Tests: Nigrosin is tested in Dorner's spore stain, using a 2 to 4 day old culture of some rapid spore-former such as *Bacillus cereus*.

A heavy suspension of the organism is made in 2 to 3 drops of distilled water in a small test tube, and an equal quantity of freshly filtered Ziehl's carbol fuchsin is added. This mixture is allowed to stand in a boiling water bath 10 minutes or longer. On a cover slip or slide, one loopful of the stained preparation is mixed with a loopful of a 5-10% aqueous nigrosin. (This solution must be filtered before use, and may be kept indefinitely if preserved with a few drops of formalin). It is smeared as thinly as possible and allowed to dry fairly rapidly. A good sample shows the spores red, the vegetative cells unstained, and the background dark gray.

THE DIPHENYL METHANE DERIVATIVES

Auramine O, C. I. No. 655.

Identification: Auramine O has the empirical formula $C_{17}H_{22}N_3Cl$. It is identified by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 5 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 430–434 mμ; ratio P–15/P+15 is from 1.02 to 1.04.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 127. D-peak (color density at peak) measured on dye solution described under "Identification".

Samples of this stain must contain not less than 80% anhydrous dye.

Biological Tests: Auramine O is tested for acid-fast bacteria in sputum. Smears from sputum are air-dried and are stained 2–3 min. in the staining solution (auramine O, 0.1 g.; liquified phenol, 3 ml.; distilled water, 97 ml.), washed in tap water, destained 3–5 min. in freshly prepared solution containing 100 ml. 70% ethyl alcohol, 0.5 ml. conc. HCl, 0.5 g. NaCl and dried. The smears are examined under a monocular microscope, using 8 mm. dry objective and a 20× ocular. Illumination should be a low voltage, high amperage microscope lamp, supplied with a blue (ultraviolet transmitting) filter, and a complementary yellow filter for the ocular. Acid-fast bacteria should be bright yellow, fluorescent, other organisms not visible and background nearly black.

THE TRI-PHENYL METHANE DERIVATIVES

Malachite Green, C. I. No. 657

Identification: Malachite green is the chloride or oxalate of p, p′-tetramethyldiamino-triphenylcarbinol anhydride, $C_{23}H_{25}N_2Cl$ or $2C_{23}H_{25}N_2 + 3C_2H_2O_4$. The method of identification is as follows: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 3 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 617–619 mμ; ratio P–15/P+15 is from 1.05 to 1.11. To differentiate between the chloride and the oxalate, dissolve approximately 0.1 g. of the dye in 50 ml. of cold water and precipitate the dye base by the addition of a slight excess of dilute NaOH. Filter off the base and divide the filtrate into two parts. Heat one part to boiling and add dilute $Ca(OH)_2$ solution. A fine white crystalline precipitate indicates that the original dye was the oxalate. Make the other portion of the filtrate slightly acid with dilute HNO_3 and if a copious

white precipitate is obtained upon addition of dilute $AgNO_3$, the original dye contained chloride.

Method of Analysis: Dissolve 200 mg. of dye in 120 ml. of distilled water, add 50 ml. alcohol, 10 ml. of glacial acetic acid and 30 ml. of 30% sodium tartrate solution, heat to boiling and titrate slowly with 0.05 *N* $TiCl_3$ to a light straw color. The following data are used in calculating the percentage of anhydrous dye in the sample:

(1) Molecular weight, $C_{23}H_{25}N_2Cl$	364.903
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	54.810
(2) Molecular weight, $2C_{23}H_{25}N_2 + 3C_2H_2O_4$	928.000
Hydrogen equivalents per mol of dye	4.
ml. of N/10 $TiCl_3$ per gram of dye	43.057

Samples of this stain should contain not less than 75% anhydrous dye (if the chloride) or not less than 90% dye (if the oxalate).

Biological Tests: Malachite green is tested for use as a counterstain in plant histology. Botanical material (preferably plant pathological material), after fixation in a suitable fixative (e. g., Flemming's, Carnoy's or Farmer's fluid) and embedding in paraffin, is sectioned and stained with 0.5% malachite green in 95% alcohol, distilled water, or in clove oil, either with or without previous staining in 1% aqueous safranin. The malachite green is applied for one minute when used alone, or for 20 seconds following 20 minutes of the safranin. When used alone, the stain should reveal clearly all such histological elements as the cell walls, endodermis, bast, cytoplasm, nuclei, and chloroplasts. When used following safranin on plant pathological material, the host nuclei, xylem, and cutinized walls, as well as the nuclei of the infecting fungus, should appear red. The cytoplasm and cellulose walls of the host should appear green.

It is also tested in the Schaeffer and Fulton spore stain for bacteria and in Conklin's modification of the method. For the former, bacterial smears are fixed in a flame and flooded with 5% aqueous malachite green for 30–60 seconds, then heated to steaming three or four times. They are washed in water 30 seconds and 0.5% aqueous safranin is added for 30 seconds; they are then washed and blotted dry. The spores should be green, the rest of the cells red.

For the latter method, bacterial smears are fixed in a flame and flooded with 5% aqueous malachite green and allowed to steam 10 min. The slides are washed in running water 30 seconds and counterstained 1 minute with 5% aqueous mercurochrome. The spores should be green, the rest of the cells red.

Brilliant Green, C. I. No. 662

Identification: Brilliant green is the acid sulfate of p, p′-tetraethyldiaminotriphenylcarbinol anhydride, $C_{27}H_{33}N_2SO_4H$. The method of identification is as follows: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 3 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 628–631 mμ; ratio P–15/P+15 is from 0.94 to 1.08.

Method of Analysis: Dissolve 100 mg. of dye in 100 ml. of 95% alcohol, add 100 ml. of distilled water and 15 g. sodium acid tartrate, heat to boiling and titrate with 0.05 *N* $TiCl_3$ to a pale yellow endpoint. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	482.618
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	41.440

For certification, samples of this stain must contain not less than 85% anhydrous dye.

Biological Tests: Brilliant green is tested as to its suitability for determining the presence of the colon organism in drinking water. The object of the dye is to prevent formation of gas by the bacillus of gas gangrene, *Bacillus Welchii*. For this purpose standard methods prescribe a medium containing 2% dried oxgall with a 1/75,000 dilution of brilliant green after addition to the water to be tested. In testing a dye sample, varying amounts of brilliant green are added to the bile medium for one series of tests, using a sufficient variety of solutions to be certain of growth in the most dilute and absence of growth in the most concentrated. A second series of tests is set up with varying dilutions of pure cultures of the colon organism (*Escherichia coli*) on the standard medium as above mentioned, while another comparison is made in standard lactose broth. A satisfactory sample should allow 10% gas production in 1 to 3 days by the colon organism, but not by *Bacillus Welchii* at the dilution called for in the standard medium. The object of the long series of tests is to check up on the delicacy of the medium as used, and on the effect of varying the quantities of brilliant green.

It is also tested as a bacteriostatic agent by the following technic:

Make three daily transfers at 37° C. of a spore-former (e.g. *Bacillus cereus*) and of two members of the colon-typhoid group (including *Escherichia coli*) into a broth containing 1% peptone and 1% lactose. As soon as distinct turbidity appears in the third transfer (3–6 hours incubation), make a microscopic count of the organisms, with the use of a hemocytometer; (it is desirable to have the count between 4 and 20 million per milliliter). Dilute with the above-mentioned broth so that the concentration of bacteria is approximately 200 per milliliter. Meanwhile make a 0.01% solution of the sample to be tested and of a check batch of brilliant green (known to have the correct bacteriostatic titre) by boiling 10 mg. dye in a 100 ml. volumetric flask not quite full of distilled water and bringing up to the volume mark after cooling over night. From this flask prepare 0.001%, 0.0001% and 0.00001% dilutions. Sterilize the flasks containing these solutions and thereafter handle them aseptically. Fill a series of test tubes with 7.5 ml. of a broth containing 1.33% peptone and 1.33% lactose, and divide into three lots, one intended for each of the three test organisms; sterilize in the autoclave. To each of these three series of tubes add varying quantities of the dye by introducing, with a sterile pipet, 0.2–2.0 ml. of the four above-mentioned dilutions; then bring the total volume in each tube up to 9.5 ml. by adding sterile distilled water aseptically, and mixing thoroughly. (In this way final dilutions from 1:50,000 to 1:500,000,000 may be obtained, although a partial set of them can be selected for each particular organism, from 1:5,000,000 to 1:100,000,000 for the spore-former and from 1:50,000 to

1:2,000,000 for the others). Inoculate each set of the tubes containing broth and diluted dye with the proper culture, using 0.5 ml. of the diluted culture (i.e. approximately 100 organisms) per tube. Mix each tube well and incubate at 37° C. Examine for growth on the 1st, 2nd and 4th days.

A satisfactory sample should show bacteriostatic action on all three organisms sufficiently like that of the check sample so that the first dilution permitting growth will not be more than two stages apart from it in the above series of final dilutions.

Fast Green FCF

Identification: Fast green FCF is the disodium salt of p, p′-dibenzyldiethyldiamino-p″-hydroxytriphenylcarbinol trisulfonic acid anhydride, $C_{37}H_{34}O_{10}N_2S_3Na_2$. Identification is by the following method: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 3 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 624–625 mμ; ratio P–15/P+15 is from 1.02 to 1.09.

Method of Analysis: Dissolve 200 mg. of dye in 200 ml. of distilled water, add 3 g. of sodium acid tartrate, heat to 70° C and titrate with 0.05 *N* $TiCl_3$. The end point varies in color from yellow to reddish brown. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	808.832
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	24.727

Samples of this stain should contain not less than 85% anhydrous dye.

Biological Tests: Fast green is tested for use as a cytological counterstain. Sections of root tips or buds are fixed in Flemming's fluid or in CRAF and are stained in 1% aqueous safranin for from 30 to 50 minutes, rinsed in distilled water and differentiated with 0.2% fast green in 95% alcohol until the chromatin and nucleoli remain red. The slides are then passed through absolute alcohol to xylene and mounted in balsam. Chromosomes and other chromatin bodies as well as nucleoli and lignified walls should appear bright red, while the spindles, cellulose walls, and cytoplasm are green.

Light Green SF Yellowish, C. I. No. 670

Identification: Light green SF yellowish is the disodium salt of p, p′-dibenzyldiethyl-diaminotriphenylcarbinol trisulfonic acid anhydride, $C_{37}H_{34}N_2O_9S_3Na_2$. Identification is by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 5 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 629–634 mμ, ratio P–15/P+15 is from 1.04 to 1.09.

Method of Analysis: Dissolve 200 mg. of dye in 200 ml. of distilled water, add 10 g. sodium acid tartrate, heat to 70°C. and titrate with 0.05 *N* $TiCl_3$. The end point is a sharp change from light green to yellow. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	792.832
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	25.226

Samples of this stain should contain not less than 70% anhydrous dye.

Biological Tests: Light green is tested by the same procedure as fast green.

Basic Fuchsin, C. I. No. 677

Identification: Stains marketed under the name of basic fuchsin may be either the chloride or acetate of pure* pararosanilin, or mixtures of it with the higher homologs. Rosanilin (the base) is triaminodiphenyltolylcarbinol. Its chloride has the formula, $C_{20}H_{20}N_3Cl$, its acetate is $C_{20}H_{20}N_3 \cdot C_2H_3O_2$. Pararosanilin (the base) is triaminotriphenylcarbinol. Its chloride has the formula, $C_{19}H_{18}N_3Cl$, and its acetate is $C_{19}H_{18}N_3 \cdot C_2H_3O_2$. The method of identification is: Dissolve 50 mg. of the sample in 125 ml. of 95% alcohol and then dilute to 250 ml. with distilled water. Dilute 3 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maxima; pararosanilin 545–546 mμ; rosanilin 549–550 mμ; ratio P–15/P+15 for pararosanilin is 1.26 to 1.34, for rosanilin 1.16 to 1.35. To test for acetate, add 1 ml. of 6*N* H_2SO_4 to 2.0 g. of the dye dissolved in 5 ml. of distilled water, and heat. The odor of acetic acid escaping from the hot mixture indicates the presence of acetate in the original sample; if not present the sample can be assumed to be the chloride.

Decolorization Test: Dissolve 0.5 g. of the dye in 100 ml. of boiling distilled water, cool to 50° C., filter into a small flask and add 10 ml. of N hydrochloric acid to the filtrate. Add 0.5 g. of potassium metabisulfite, $K_2S_2O_5$, shake until dissolved, stopper tightly and allow to stand in the dark for 12–18 hours. The solution is colorless, or not more than pale yellow. (A yellowish orange, yellowish brown or brown solution, especially in the presence of a dark sediment, indicates the poorer grades of fuschin.)

Method of Analysis: Spectrophotometric. Percent dye (pararosanilin acetate) = D-peak × 129. Percent dye (rosanilin chloride) = D-peak × 122. D-peak (color density at peak) measured on dye solution described under "Identification". The following data are used in calculating the percentage of anhydrous dye in the original sample.

Rosanilin:	*Chloride*	*Acetate*
Molecular weight	337.841	361.428
Hydrogen equivalents per mol of dye	2.	2.
ml. of N/10 $TiCl_3$ per gram of dye	59.200	55.336

Pararosanilin:	*Chloride*	*Acetate*
Molecular weight	323.815	347.402
Hydrogen equivalents per mol of dye	2.	2.
ml. of N/10 $TiCl_3$ per gram of dye	61.673	57.670

*"Pure" is used here in the sense of freedom from homologous dyes, and not in the sense of freedom from such impurities as inorganic salts, colorless organic substances, and sometimes subsidiary dyes.

For certification, samples of this stain should contain not less than 88% anhydrous dye.

Biological Tests: Basic fuchsin is tested for several purposes: (1) For use as a stain for the tubercle organism; (2) for use in the Endo medium for distinguishing between the coli and aerogenes types of bacteria; (3) for its bacteriostatic action; (4) for use in the Feulgen stain.

(1) In testing as a stain for the tubercle organism, smears are covered with carbol fuchsin (1 part of 3% alcoholic fuchsin to 9 parts of 5% aqueous phenol) and heated on a water bath for 3-5 minutes. They are then rinsed in tap water and differentiated in 70% alcohol containing 3% hydrochloric acid until practically no red color remains visible to the naked eye. They are again rinsed in tap water; rinsed once more and counterstained with dilute (i. e., about 0.1%) aqueous methylene blue for 1 minute. The slides are then rinsed in tap water and dried and examined under the microscope. The tubercle organisms should be distinctly red, while other bacteria, leucocytes and debris appear blue.

(2) In testing for use in the Endo medium, three separate solutions of the sample to be tested are prepared: saturated alcoholic; 1% alcoholic; and 3% alcoholic. Of these solutions, 0.5 ml. of the first and 1 ml. of each of the others are mixed separately with 0.125 g. of anhydrous sodium sulfite dissolved in 5 ml. of hot distilled water. The tests are carried out separately with each of these three solutions; good results must be obtained with at least one of them. The solution should be a faint pink or straw color, or sometimes light brown, but without noticeable precipitate. This decolorized solution is added to 100 ml. of melted lactose agar of the standard formula. The color of this medium should then be a light pink, which fades almost entirely upon cooling. Before cooling it is poured into a Petri dish and allowed to harden. The surface is then streaked in parallel lines with *Escherichia coli* and *Aerobacter aerogenes* and allowed to incubate for 24 hours. A good sample should show red growth with a strong metallic sheen for the former organism, pink growth without metallic sheen for the latter, and no reddening of the medium except where growth has occurred.

(3) In testing for its bacteriostatic action, the same methods are followed as in the case of brilliant green (p. 292) except that the dilutions for the spore-former are from 1:100,000 to 1:1,000,000 and for the others 1:2,500 to 1:40,000.

(4) In testing for its behavior in the Feulgen stain, the procedure followed is to test the stain on properly prepared sections from which the paraffin has been removed in the usual manner; these sections are rinsed in cold *N* HCl, placed 4-5 minutes in *N* HCl at 60° C., and then rinsed in cold *N* HCl, and finally in distilled water. If the sections are of animal tissue they are then stained 2 hours, or if plant tissue 3-5 hours, in the following solution: 0.5 g. of the sample to be tested is dissolved by pouring over it 100 ml. boiling distilled water, then shaken thoroughly and cooled to 50° C., filtered, 0.5 g. $K_2S_2O_5$ and 10 ml. *N* HCl are added; the solution is allowed to stand in the dark 24-48 hours; a good sample should then be almost completely decolorized, at the most showing a very faint straw color. The sections, after remaining for the specified time in this decolorized solution, are drained and passed for 10 minutes each into three successive baths containing *N* HCl, 10% $K_2S_2O_5$, and distilled water in the proportion of 5:5:100 by volume (carrying out

this step in closed jars); they are then rinsed in distilled water and counterstained in 1% aqueous orange G (for animal tissue) or 0.5% alcoholic fast green FCF or light green (for plant tissue), dehydrated, cleared and mounted in balsam. A good basic fuchsin should show almost complete decolorization of the solution after standing 24 or at the most 48 hours, and on staining should give a reddish violet color to the chromosomes. A sample which does not thus decolorize, however, need not be rejected if a clear solution can be obtained by the following procedure: To 85 ml. distilled water, add 1 g. of sample, 1.9 g. $Na_2S_2O_5$, 15 ml. *N* HCl. Place in a bottle with 50–60 ml. free air space above the fluid. Shake for 2 hours at intervals or on a mechanical shaker. Add 500 mg. fresh activated charcoal, and shake for 2 minutes. Filter on a plaited hard filter. Solution should be colorless.

"SPECIAL" BASIC FUCHSIN FOR FLAGELLA STAINING

Samples sold under this designation are presumed to be mixtures of pararosanilin acetate and chloride in the proportion of 3 : 1 by weight, as recommended by Leifson (1951).

Biological Tests: They are tested by Leifson's method: Treat slides 1 week in K_2CrO_4 in conc. H_2SO_4, wash thoroughly with final rinsing in distilled water, drain dry, hold in colorless flame for few seconds, cool, make heavy wax line across middle and along margin to distal half. Place a loopful of sediment from a centrifuged 24-hour broth culture of a motile species of bacteria at end of slide; tilt, causing liquid to flow to opposite wax line; air dry. Add quickly 1 ml. of stain mixture (equal proportions of the following 3 solutions mixed together: 1.5% NaCl in distilled water; 3% tannic acid in distilled water; 1.2% of sample in 95% alcohol). Leave at room temperature 10 min., rinse slide with tap water under faucet, but do not pour stain off before rinsing and drain dry. Satisfactory sample should show the flagella bright red.

Gentian Violet

Dyes furnished under this label are frequently poorly defined mixtures of methylated rosanilins, of the type usually designated as methyl violet. In some cases crystal violet is marketed under the name gentian violet, and at times products of this nature have both names on the label. Since crystal violet is more satisfactory for bacteriological work and for those histological purposes where a deep blue-violet is required, and methyl violet 2B more desirable in histological procedures where a reddish-violet is called for, users should specify which of the two is desired and should avoid entirely the term gentian violet. It is hoped that when users have been sufficiently educated, distributors of stains can be induced to label these violets methyl or crystal, as the case may be, and to abandon the meaningless name gentian violet.

When a dye is submitted under the name gentian violet, the nature of the dye which predominates is determined spectrophotometrically, and the analysis and biological tests are made as described under that dye. (See methyl violet, C. I. No. 680, and crystal violet, C. I. No. 681.)

Methyl Violet 2B, C. I. No. 680

Identification: Methyl violet 2B is a mixture of the more highly methylated fuchsins, principally pentamethylpararosanilin chloride, that is, the chloride of pentamethyltriaminotriphenylcarbinol anhydride. For purposes of estimation the formula $C_{24}H_{28}N_3Cl$ is employed. The following method of identification is used: Dissolve 50 mg. of sample in 250 ml. of 50% alcohol. Dilute 2 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 583–587 mμ; ratio P–15/P+15 is from 1.07 to 1.18.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 207. D-peak (color density at peak) measured on dye solution described under "Identification".

Samples of this stain should contain not less than 75% anhydrous dye.

Biological Tests: Methyl violet 2B is tested by the same procedure as crystal violet, C. I. No. 681. Results should be the same except that the violet color obtained is of a redder hue.

Crystal Violet, C. I. No. 681

Identification: Crystal violet is the chloride of hexamethylpararosanilin, that is, hexamethyltriaminotriphenylcarbinol anhydride, $C_{25}H_{30}N_3Cl$. Identification is by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 2 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 589–593 mμ; ratio P–15/P+15 is from 1.04 to 1.19.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 209. D-peak (color density at peak) measured on dye solution described under "Identification."

For certification, samples of this stain must contain not less than 88% anhydrous dye.

Biological Tests: Crystal violet is tested in four different procedures: (1) the Gram stain; (2) the Flemming triple stain; (3) followed by iodine in the staining of cytological preparations; (4) for its bacteriostatic action.

(1) In testing crystal violet for staining bacteria by the Gram technic, the sample is made up in a 0.5% aqueous solution, without the use of anilin oil or any other mordant, and bacterial smears are stained by the following procedure: Crystal or gentian violet 1 minute, tap water 1–5 seconds, Lugol's iodine solution 1 minute, wash in tap water and blot dry, 95% alcohol 30 seconds, 0.25% safranin solution 10 seconds, tap water 1–5 seconds. In making the test, a weakly Gram-positive organism and a Gram-negative organism are tested; the former should appear blue, the latter red.

(2) In testing the sample in the Flemming stain, the following procedure is followed: Root tips or buds, fixed in Flemming's fluid or CRAF, are embedded in paraffin and sectioned. Sections are stained for 20 minutes to 1 hour in 1% aqueous safranin, rinsed in distilled water (sometimes differentiated in weakly acidulated alcohol and then rinsed in distilled water), stained from 2 to 30 minutes in a 1% solution of the sample being tested, again rinsed in distilled water, dehydrated in 95% and absolute alcohol, differentiated in a 0.25% solution of orange G in clove oil about 15 minutes, cleared in xylene and mounted in Canada balsam. A good

stain should show red chromosomes and nucleoli, while metabolic chromatin should stain a deep purple on an orange cytoplasm.

(3) In the test with iodine, paraffin sections of root tips or buds (fixed in Navashin's fluid or one of its modifications) are stained for 3 to 10 minutes in a 1% aqueous solution of the dye, rinsed in distilled water, and treated for 15 to 50 seconds in equal parts of 1% iodine in 80% alcohol and 1% potassium iodide in 80% alcohol. They are next rinsed in absolute alcohol and differentiated in clove oil and cleared in xylene. By this technic, chromatin and nucleoli should stand out a deep violet on a colorless protoplasm.

(4) In testing for its bacteriostatic action, the same methods are followed as in the case of brilliant green (p. 312) except that the dilutions for the spore-former should be 1:1,000,000 to 1:40,000,000 and for the others 1:5,000 to 1:250,000.

Methyl Green, (Ethyl Green) C. I. No. 685

Identification: This methyl green is the zinc chloride double salt of ethylhexamethylpararosanilin chlorobromide, $C_{27}H_{35}N_3ClBr+ZnCl_2$. Identification is by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 10 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 630–631 mμ; ratio P–15/P+15 is from 0.91 to 0.95. Dilute solution just prior to reading because of instability of solution. Take peak readings first then ratio points as rapidly as possible.

Method of Analysis: Dissolve 200 mg. of dye in 100 ml. of 95% alcohol, add 75 ml. of distilled water and 25 ml. of 30% sodium tartrate solution, heat to boiling and titrate with 0.05 *N* $TiCl_3$ to a greenish-yellow endpoint. The following data are used to calculate the percentage of anhydrous dye in the sample:

Molecular weight	652.241
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	30.617

For certification, samples of this stain must contain not less than 65% anhydrous dye.

Biological Tests: Methyl green is tested in staining gonorrhoeal smears by the Pappenheim-Saathof stain. The smears from pus are prepared and dried in the air. They are stained 15 seconds without heat in the methyl-green-pyronin stain (methyl green, 1 g.; pyronin Y or B, 0.25 g.; 95% ethyl alcohol, 5 ml.; glycerol, 20 ml.; 2% aqueous phenol, 100 ml.) washed in distilled water, blotted dry and examined. The cocci should be bright red and the nuclei green (not purplish).

It is also tested as counterstain for Bismarck brown by the procedure given on page 300. With a good sample, all the nuclei of the cells stain green.

Acid Fuchsin, C. I. No. 692

Identification: Acid fuchsin should be the disodium salt of the trisulfonic acid of rosanilin or pararosanilin or mixtures of these two. Some samples may contain the mono or disodium salts of the disulfonic acid or the trisodium salt of the trisulfonic acid, but if such is the case they are not nearly as satisfactory as those which con-

tain only the disodium trisulfonate. Samples of acid fuchsin are considered for analytical purposes as a mixture of equal parts of trisulfonated rosanilin and pararosanilin, unless specific information concerning the character of the fuchsin used for the starting material has been obtained from the dye manufacturer. Identification is by the following method: Dissolve 50 mg. of sample in 250 ml. of distilled water. Dilute 5 ml. of this solution and 5 ml. of 0.1 *N* HCl to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum for pararosanilin 540–542 mμ, for rosanilin 543–546 mμ; ratio P–15/P+15 for pararosanilin is from 1.13 to 1.26, for rosanilin from 1.11 to 1.15.

Andrade Indicator: Acid fuchsin is tested as to its behavior in the Andrade indicator as follows: A 0.2% solution of the dye in 100 ml. of distilled water is decolorized by adding normal NaOH a little at a time. Complete decolorization should be brought about without the use of more than 25 ml. of the sodium hydroxide. (The quantity of NaOH required should be stated on the label.)

Method of Analysis: Spectrophotometric. Percent dye (for pararosanilin) = D-peak × 142. Percent dye (for rosanilin) = D-peak × 151. D-peak (color density at peak) measured on dye solution described under "Identification".

For certification, samples of this stain must contain not less than 55% anhydrous dye.

Biological Tests: Acid fuchsin is tested in the Van Gieson connective tissue stain on animal tissue fixed in formalin or other suitable fixing fluid and embedded in paraffin. The paraffin is removed in the usual manner; the sections are stained deeply in alum-hematoxylin or Weigert's iron-hematoxylin, washed in distilled water and stained 3-5 minutes in Van Gieson's solution, (5 ml. of 1% aqueous acid fuchsin and 100 ml. of saturated aqueous picric acid). They are then quickly washed in tap water, differentiated in 95% alcohol, dehydrated, cleared and mounted in balsam. The tissue elements stained by the acid fuchsin should show a clear red, contrasting well with hematoxylin and picric acid. A good sample does not show great tendency to fade.

Acid fuchsin is also tested in the anilin blue connective tissue stain on animal tissue fixed in Zenker's fluid and embedded in paraffin. The paraffin is removed in the usual manner and the sections are stained in a 0.5% aqueous acid fuchsin for 1–5 minutes or longer, depending on the freshness of the tissue. The sections are transferred directly to the following solution (anilin blue W.S., 0.5 g.; orange G, 2.0 g.; 1% aqueous phosphotungstic acid, 100 ml.) for 10 minutes or longer, dehydrated in several changes of 95% alcohol, then absolute alcohol, cleared in xylene and mounted in balsam. The same criteria are used in judging a good sample with this technic as with the Van Gieson stain.

Anilin Blue, Water Soluble, C. I. No. 707

Identification: Anilin blue consists of mixtures of the various sulfonation products of variable mixtures of phenylated rosanilin and pararosanilin, usually the latter. The method of identification is as follows: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 10 ml. of this solution and 10 ml. of 0.1 *N* HCl to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 595–610 mμ; ratio P–15/P+15 is from 0.98 to 1.09.

Decolorization Test: To 100 ml. of a 0.01% solution of the dye add 2 ml. of *N* NaOH. The color should turn red immediately, fading after 10 minutes to a straw color, and after 20 minutes should become almost colorless. (Some good samples become colorless in 10 minutes.) Addition of a few drops of normal acid should restore the blue color almost instantly.

Method of Analysis: Dissolve 200 mg. of dye in 175 ml. of distilled water, add 25 ml. of pH 4.5 acetate buffer, heat to boiling and titrate with 0.05 *N* $TiCl_3$. The end point will vary, depending on the sample, from yellow to dark yellow-green, and is generally easy to detect. Because of the uncertain composition of anilin blue, no attempt is made to determine the dye content. Specifications have been made which place the minimum $TiCl_3$ consumption per gram of dye at 12.0 ml. of 0.1 *N* solution.

Biological Tests: Anilin blue is tested in Mallory's connective tissue stain on animal tissue fixed in Zenker's fluid and embedded in celloidin or paraffin. The procedure followed is the one given under acid fuchsin, p. 319. With a good sample, the collagen fibrils, reticulum, amyloid, and mucus stain blue.

THE FLUORAN DERIVATIVES

The fluoran derivatives are readily reduced by titanous chloride and excellent checks are obtained in duplicate reductions, but due to the heterogeneous character of some of the commercial samples of the halogenated dyes of this group, large errors in the indicated dye content are encountered. It has been found that the dye content of these samples may be determined more accurately and conveniently by the conversion of the dye into the form of the insoluble color acid.

Certain members of this group are used as biological stains, but as there is considerable difference in the purpose for which the various members of the group are employed, different biological tests are necessary for each of the dyes listed. The biological tests employed at the present time are given for all these dyes, with the single exception of fluorescein.

The following methods, employed for the standardization of the fluorans, include (1) the identification or qualitative examination; (2) the quantitative analysis; and (3) the biological tests for each individual dye.

Eosin Yellowish, C. I. No. 768

Identification: Eosin yellowish (eosin Y) is the sodium salt of tetrabromofluorescein, $C_{20}H_6O_5Br_4Na_2$. The following method of identification is employed: Dissolve 50 mg. of dye in 250 ml. of distilled water. Dilute 5 ml. of this solution and 2 ml. of 1% Na_2CO_3 solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 515–517 mμ; ratio P–15/P+15 is from 1.21 to 1.77.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 147. D-peak (color density at peak) measured on dye solution described under "Identification".

For certification, samples must contain not less than 80% anhydrous dye.

Biological Tests: Eosin Y is tested for three biological purposes: as a counterstain against hematoxylin, as a constituent of Wright's stain, and in the eosin-methylene-blue medium.

In Heidenhain's hematoxylin, Zenker's fixed material is used and embedded in paraffin. The paraffin is removed in the usual manner. Sections are mordanted in 1.5–4% aqueous $NH_4Fe(SO_4)_2 \cdot 12H_2O$ (violet crystals) for 30 minutes to 3 hours, rinsed in tap water, stained 1–3 hours in 0.5% solution of hematoxylin in distilled water, ripened 4–5 weeks, rinsed again in tap water and differentiated in the above iron alum solution, controlling the differentiation by microscopic examination. They are then washed in running water about 5–10 minutes, and are counterstained in a 0.1% strength of eosin Y in 25% alcohol for 2–5 minutes, dehydrated, cleared and mounted in balsam. By this technic, the nuclei should be black and the cytoplasmic structures, pink.

In testing its behavior as a constituent of Wright's stain the formula given on page ID_3-4 of Staining Procedures is employed and the compound stain, after dissolving in methyl alcohol, is applied to blood smears for one to three minutes without diluting and is then diluted on the slide in distilled water with two to six minutes application of the diluted stain. The smear is washed in distilled water and dried and examined under the microscope. In judging a good Wright's stain, special attention is given to the question of whether the red cells show a desired yellow pink or whether all the granules of the various leucocytes are differentially stained.

The eosin-methylene-blue medium is prepared as follows: 1 g. peptone, 0.2 g. K_2HPO_4, 1½ g. agar and 100 ml. distilled water. Dissolve by heat and add 5 ml. of 20% aqueous lactose, 2 ml. of 2% aqueous eosin Y and 2 ml. of 0.5% aqueous methylene blue. After sterilizing, pour into Petri dishes and inoculate with *Escherichia coli* and *Aerobacter aerogenes* and allow to incubate for 24 hours. A good sample should show red growth with a strong metallic sheen for the former organism, pink growth without metallic sheen for the latter and no reddening of the medium except where growth has occurred.

Ethyl Eosin, C. I. No. 770

Identification: Ethyl eosin is the potassium (or sodium) salt of the ethyl ester of tetrabromofluorescein, $C_{22}H_{11}O_5Br_4K$ (or Na). The method of identification is as follows: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 5 ml. of this solution and 2 ml. of 1% Na_2CO_3 solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 531–533 mμ; ratio P–15/P+15 is from 1.27 to 1.62.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 130. D-peak (color density at peak) measured on dye solution described under "Identification".

For certification, samples must contain not less than 78% anhydrous dye.

Biological Tests: Ethyl eosin is tested for the demonstration of Negri bodies in the central nervous system of rabid animals. Pieces of the hippocampus major, 3–5 mm. in thickness, are placed between squares of ordinary writing paper (cut end next to the paper) and are immersed in acetone 2½–6 hours. The paper is removed and the fixed tissue is placed in fresh paraffin at 58–60° C. for 4 hours or overnight. Sections are cut 5 μ thick, floated onto glass slides and fixed by gentle heat over Bunsen burner, and placed in oven for 45 minutes at 58–60° C. They are washed in two changes of xylene and passed through two changes of absolute alco-

hol and two changes of 95%, then 70% alcohol to distilled water; then stained 2 minutes in 1% ethyl eosin in 95% ethyl alcohol, adjusted to pH 3.0 with N/10 HCl. (If this solution fails to stain, about 1% acetic acid is added.) They are then washed in distilled water, stained 30 seconds in a solution of 0.3 g. methylene blue in 30 ml. 95% ethyl alcohol and mixed with 100 ml. distilled water, adjusted to pH 5.6 by adding 2 ml. of acetate-acetic acid (sodium acetate, 25 g.; glacial acetic acid, 5 ml.; distilled water, 250 ml.) to 60 ml. of fluid, washed in distilled water and differentiated in water acidulated with acetic acid until the sections become brownish red, rinsed in distilled water, dehydrated, cleared in xylene and mounted in balsam. By this technic, the nerve cells are stained blue and the Negri bodies terra cotta to cardinal red.

Eosin Bluish, C. I. No. 771

Identification: Eosin bluish (eosin B) is the sodium salt of dibromodinitrofluorescein, $C_{20}H_6N_2O_9Br_2Na_2$. The method of identification is: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 5 ml. of this solution and 2 ml. of 1% Na_2CO_3 solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 516–519 mμ; ratio P–15/P+15 is from 1.00 to 1.10.

Method of Analysis: Spectrophotometric. Percent dye = D-peak × 226. D-peak (color density at peak) measured on dye solution described under "Identification".

Samples of this stain should contain not less than 85% anhydrous dye.

Biological Tests: Eosin B is tested as a counterstain for hematoxylin by the following technic: Material fixed in either Bouin's fluid or Zenker-formol is embedded in paraffin and cut 10 μ in thickness. Sections are mounted on slides with Mayer's albumen fixative. They are then run down to distilled water, stained for from 5 to 10 minutes in Mayer's hemalum, dipped once or twice in tap water, then holding them in forceps dipped into the solution of eosin once, twice or three times, dehydrated and mounted.

The solution of eosin is 0.5% solution of the stain in 20% alcohol. If it overstains, dilute with distilled water. In going quickly from Mayer's hemalum into the eosin a precipitate is formed in the latter and the color is also precipitated into the tissue so that it does not wash out readily.

Eosin B is also tested in Mallory's phloxine-methylene-blue stain. Zenker-fixed material is embedded in paraffin. The paraffin is removed in the usual manner and the material is stained 20 minutes or longer in a 2% aqueous eosin B (which is substituted for the 5% phloxine). It is washed in distilled water; stained 30 minutes in borax methylene blue (methylene blue, 1 g.; borax, 1 g.; distilled water, 100 ml.; diluted to 10 times its volume with distilled water) by pouring the solution off and on several times; washed in distilled water; differentiated and dehydrated in a dish of 95% alcohol containing a few drops of 10% alcoholic collophonium (rosin). The sections are kept in constant motion so that the decolorization is uniform. Staining is controlled under the microscope; when the pink color has returned to the section and the nuclei are still a deep blue, the dehydration is finished quickly with absolute alcohol, sections are cleared and mounted in balsam. The cytoplasm should stain pink in contrast to the blue of the nuclei.

Erythrosin B, C. I. No. 773

Identification: Erythrosin is the sodium salt of tetraiodofluorescein, $C_{20}H_6O_5I_4Na_2$. Identification is by the following method: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 5 ml. of this solution and 2 ml. of 1% Na_2CO_3 solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 524–526.5 mμ; ratio P–15/P+15 is from 1.15 to 1.41.

Method of Analysis: Dissolve an accurately weighed sample of about 0.5 g. of the dye to be examined in approximately 500 ml. of water, heat to boiling and add slowly with constant stirring, 2 or 3 ml. of 6 N hydrochloric acid. Cool the hot solution to room temperature and allow to stand at least 1 or 2 hours. Filter the precipitated color acid on a weighed Gooch crucible, thoroughly wash with a 0.2% solution of hydrochloric acid, dry at 110° C., and weigh. Calculate the dye content of the original sample from the weight of the color acid by means of the following formula:

$$\frac{\text{Wt. of Color Acid} \times 879.922 \times 100}{835.944 \times \text{Wt. of Original Sample}} = \text{per cent of anhydrous dye.}$$

Note: 879.922 = Molecular weight of erythrosin.
835.944 = Molecular weight of the color acid of erythrosin.

Samples of this stain should not contain less than 80% anhydrous dye.

Biological Tests: Erythrosin is tested in the Jackson stain (1926) for plant anatomy. In employing this procedure as a test for erythrosin, plant buds or other botanical material containing both lignified and non-lignified cell walls are fixed in one of the usual fixatives, embedded in paraffin and sectioned. After removing the paraffin they are rinsed with absolute alcohol, then 95% alcohol, and stained 15–30 minutes in 1% aqueous crystal violet. Then, after quickly rinsing in distilled water and dehydrating, they are differentiated 1–5 minutes in a saturated solution of erythrosin in clove oil, cleared in xylene-alcohol, 1:1, passed through xylene and mounted. By this method the non-lignified tissues should stain with erythrosin and the lignified walls with crystal violet.

Phloxine B, C. I. No. 778

Identification: Phloxine B is the sodium salt of tetrabromotetrachlorofluorescein, $C_{20}H_2O_5Cl_4Br_4Na_2$. Identification is as follows: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 5 ml. of this solution and 2 ml. of 1% Na_2CO_3 solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 547–548 mμ; ratio P–15/P+15 is from 1.38 to 1.62.

Method of Analysis: Precipitate the color acid of a weighed sample of the dye to be tested by the method outlined for the analysis of erythrosin B. From the weight of the color acid, the dye content of the original sample is calculated by means of the following formula:

$$\frac{\text{Wt. of Color Acid} \times 829.702 \times 100}{\text{Wt. of the Original Sample} \times 785.724} = \text{per cent of anhydrous dye.}$$

Note: 829.702 = Molecular weight of phloxine B.
785.724 = Molecular weight of the color acid of phloxine B.

Samples of this stain should not contain less than 80% anhydrous dye.

Biological Tests: Phloxine B is tested by the Mallory phloxine and methylene blue technic, by the procedure given in STAINING PROCEDURES, Leaflet IA, using 5% phloxine instead of 2% eosin B. A satisfactory stain is judged by the pink color of the cytoplasm, while the nuclei are colored by the methylene blue.

ROSE BENGAL, C. I. No. 779

Identification: Rose bengal is the sodium salt of tetraiodotetrachlorofluorescein, $C_{20}H_2O_5I_4Cl_4Na_2$. The following method of identification is employed: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 5 ml. of this solution and 2 ml. of 1% Na_2CO_3 solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 544–550 mμ; ratio P–15/P+15 is from 1.21 to 1.63.

Method of Analysis: The method for the determination of rose bengal is identical with that of erythrosin B, except that in the calculation of the dye content of the original sample, the following formula is to be used in place of the one given for the after dye.

$$\frac{\text{Wt. of Color Acid} \times 1017.718 \times 100}{\text{Wt. of Original Sample} \times 973.740} = \text{per cent of anhydrous dye.}$$

Note: 1017.718 = Molecular weight of rose bengal.
973.740 = Molecular weight of the color acid of rose bengal.

For certification, samples of this stain should contain not less than 80% anhydrous dye.

Biological Tests: Rose bengal is tested in Conn's technic for staining bacteria in soil. The procedure is to stain a dried drop of soil suspension made by mixing soil with 5–10 times its weight of 0.015% gelatin in distilled water. While on a flat surface over boiling water this film is stained 1 minute with 1% rose Bengal in 5% aqueous phenol, containing 0.01%, more or less, of $CaCl_2$. The amount of $CaCl_2$ is varied if unsatisfactory results are obtained with 0.01%, on the theory that increasing its concentration intensifies the action of the stain. A sample is judged satisfactory which permits a deep staining of the bacteria without sufficient coloring of the soil or dead organic matter to obscure the microörganisms. In making the test, soil known to have an unusually large number of bacteria is employed.

MISCELLANEOUS DYES

PYRONIN Y, C. I. No. 739

Identification: Pyronin Y is tetramethyldiaminoxanthenyl chloride, $C_{17}H_{19}N_2OCl$. Identification is by the following method: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 5 ml. of this solution to 250 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 547–550 mμ; ratio P–15/P+15 is 1.40.

Method of Analysis: Spectrophotometric. Percent dye = (D-peak × density) ÷ 1.58. D-peak (color density at peak) measured on dye solution described under "Identification". *Note:* 1.58 is equal to the value of Holmes and Peterson (1930).

For certification, samples of this stain must contain not less than 45% anhydrous dye.

Biological Tests: Pyronin is tested in staining gonorrhoeal smears by the Pappenheim-Saathof stain, as given under methyl green, p. 318. With a good sample, the cocci should be bright red and the nuclei green (not purplish).

Pyronin B, C. I. No. 741

Identification: Pyronin B is tetraethyldiaminoxanthenyl chloride, $C_{21}H_{27}N_2OCl$. Identification is by the following method: Dissolve 50 mg. in 250 ml. of 50% alcohol. Dilute 5 ml. of this solution to 200 ml. with 50% alcohol. Read in Beckman spectrophotometer. Absorption maximum 554–555 mμ; ratio P–15/P+15 is from 1.49 to 1.61.

Method of Analysis: Spectrophotometric. Percent dye $= \frac{\text{D-peak} \times 100.}{1.74}$ D-peak (color density at peak) measured on dye solution described under "Identification".

Samples of this stain should contain not less than 30% anhydrous dye.

Biological Tests: Pyronin B is tested by the same procedure as pyronin Y.

Indigo Carmine (Indigotine), C. I. No. 1180

Identification: Indigo carmine is the disodium salt of indigotin-5,5′-disulfonic acid, $C_{16}H_8N_2O_8S_2Na_2$. The following method of identification is used: Dissolve 50 mg. in 250 ml. of distilled water. Dilute 10 ml. of this solution to 200 ml. with distilled water. Read in Beckman spectrophotometer. Absorption maximum 608–611 mμ; ratio P–15/P+15 is 1.04.

Method of Analysis: Dissolve 200 mg. of dye in 200 ml. of distilled water, add 15 g of sodium acid tartrate, heat to boiling and titrate with 0.05 *N* $TiCl_3$ solution. The color change at the end point is from blue to reddish brown. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	466.354
Hydrogen equivalents per mol of dye	2.
ml. of N/10 $TiCl_3$ per gram of dye	42.895

Samples of this stain should contain not less than 80% anhydrous dye.

Biological Tests: Indigo carmine is tested in Shumways' modification (1926) of the Cajal stain. Young amphibian larvae fixed in Bouin's picro-formol-acetic are embedded in paraffin and sectioned. Sections are stained 20 minutes in basic fuchsin (sat. aq. sol.) after which they are rinsed in distilled water and placed in an equal mixture of the indigo carmine to be tested and picric acid (each in sat. aq. sol.) for 5 minutes. They are passed rapidly through 70% alcohol (when the sections appear red), 95% and absolute alcohol (until the sections appear green) and cleared in xylene. With a satisfactory sample muscle tissue is green and connective tissue blue, contrasting sharply with the red of the nuclei.

Carmine, C. I. No. 1239

Identification: Carmine is a cochineal product, usually furnished as the aluminum-calcium salt of carminic acid. The exact structure for carminic acid is somewhat

uncertain; the molecular formula is $C_{22}H_{20}O_{13}$. It is a strong dibasic acid with a side chain which contains four hydroxyl groups and possibly possesses a sugar-like structure, although carminic acid is not a glucoside. It contains at least one asymmetric carbon.

Method of Analysis: Due to the uncertainty of its constitution, no dye content is determined for samples of this stain.

Biological Tests: In testing carmine, Mayer's alcoholic hydrochloric acid stain is prepared by dissolving 4 g. carmine by boiling in 15 ml. distilled water with 30 drops of concentrated hydrochloric acid, then adding 95 ml. of 85% alcohol and neutralizing by adding ammonia until the carmine begins to precipitate. This is employed for staining chick or pig embryos fixed in Bouin's or Zenker's fluid. For sections, the embryos are stained in bulk 24 to 72 hours, without decolorization, embedded in paraffin, and sections are cut 10 to 15 μ thick. For *toto* mounts of both chick and pig embryos, the stain is applied for about 24 hours to secure deep staining, decolorized until rose-pink in 1% HCl in 80% ethyl alcohol, dehydrated and cleared. Small embryos are mounted in balsam and larger ones in benzyl benzoate. A good sample of carmine is indicated by the degree of nuclear staining secured.

Carmine is also tested in Schneider's aceto-carmine formula (saturated solution in boiling 45% glacial acetic acid) for staining chromosomes in smears of anthers. The anthers are squeezed out gently onto the slide. A small drop of carmine is quickly put on the anthers, and a cover slip is placed on it. It is heated gently and then examined. The result should be a dark translucent red stain, selective for chromatin, with uncolored cytoplasm.

Hematoxylin

Identification: Hematoxylin is a logwood product whose exact chemistry is not entirely understood, although its formula is assumed to be as given on p. 202. Because of this uncertainty, reliable chemical methods for its identification and analysis have not yet been devised.

Biological Tests: It is tested on paraffin sections of rabbit embryo by Delafield's, Heidenhain's, and Ehrlich's technics, as follows:

Delafield's hematoxylin is made up as follows: hematoxylin, 4 g. is dissolved in 25 ml. 95% ethyl alcohol and 400 ml. saturated aqueous $AlNH_4(SO_4)_2 \cdot 12H_2O$ is added; this is allowed to stand a week exposed to air and light; it is then filtered and 100 ml. glycerin and 100 ml. methyl alcohol is added. This solution becomes dark in 6–8 weeks after which it is tested. Sections are stained for 15 minutes in this solution after diluting with equal parts of water; they are then rinsed in tap water and immersed in fresh tap water for about 10 minutes. If the sections are still very blue, 2 drops of acidulated 35% alcohol (containing 3 drops conc. HCl to 50 ml.) are added to them on the slides and they are returned to the water. They are then passed through 95% alcohol and absolute alcohol into xylene and are mounted in balsam. A satisfactory sample should show blue nuclei.

Heidenhain's technic is as follows: The paraffin is removed from the sections in the usual manner. The sections are mordanted in 1.5–4% aqueous $NH_4Fe(SO_4)_2 \cdot 12H_2O$ for 30 minutes to 3 hours, rinsed in tap water, stained 1–3 hours in 0.5%

aqueous solution of the ripened hematoxylin, rinsed in tap water, differentiated in the above iron alum solution, controlling the differentiation by microscopic examination, washed in running water about 5–10 minutes, counterstained with 0.1% eosin Y in 25% alcohol 2–5 minutes, dehydrated, cleared and mounted in balsam. The criterion of a satisfactory sample is that the nuclei should appear black.

Ehrlich's method is as follows: Dissolve 2 g. hematoxylin in 100 ml. 95% ethyl alcohol, and add 100 ml. distilled water, 3 g. $AlNH_4(SO_4)_2 \cdot 12H_2O$, 100 ml. glycerin, and 10 ml. glacial acetic acid. Ripen by exposure to air and sunlight in a paper-capped vessel for 4–6 weeks or immediately by addition of 0.4 g. $NaIO_3$. Sections are stained 2–5 min. in this solution, washed in tap water until section appears blue, stained 1 min. in 0.5% aqueous eosin Y, rinsed in water, dehydrated in 2 changes each of 95% and 100% alcohol, cleared and mounted in balsam. A satisfactory sample should show dark blue nuclei.

Orcein

Identification: The exact formula of orcein is unknown. The following method of identification is employed: Dissolve 50 mg. in 250 ml. of 0.01 *N* NaOH. Dilute 10 ml. of this solution to 200 ml. with 0.01 *N* NaOH. Read in Beckman spectrophotometer. Absorption maximum 576–582 mμ; ratio P–15/P+15 is from 1.01 to 1.18.

Method of analysis: Since the exact formula of orcein is unknown, determination of dye content is impossible.

Biological Tests: Orcein is tested as an elastic tissue stain on Zenker's, acetone, formalin, or Bouin's fixed tissue. The paraffin is removed in the usual manner. The sections are stained 30 min. in orcein (orcein, 0.4 g. in 70% alcohol + 1 ml. conc. HCl), rinsed briefly in 70% alcohol, then in distilled water, stained 5 min. in diluted methylene blue solution (methylene blue, 1 g.; borax, 1 g.; distilled water, 100 ml.; for use, diluted with 9 vol. of distilled water), differentiated and dehydrated in a dish of 95% alcohol to which has been added a few drops of 10% alcoholic rosin, keeping the section in constant motion about 2 min.; dehydration is quickly completed with absolute alcohol, followed by clearing in xylene and mount in balsam. A satisfactory sample should show even the finest elastic fibers either a dark purple or reddish purple.

Wright Stain

Identification: Wright stain is a compound of eosin with an oxidized and partly demethylated methylene blue. Because of its complexity no satisfactory chemical methods have been yet worked out for its identification or analysis.

Biological Tests: The dye is dissolved in methyl alcohol (1 to 600) and is applied to a dried blood film for 1–3 minutes. Then 1 ml. of distilled water is added. After standing twice as long as the undiluted stain, this solution is poured off and the slide is washed with distilled water, until the thin portions of the stained film are pink. The smear is dried by blotting carefully. A satisfactory sample should show the following picture: erythrocytes, yellowish red; polymorphonuclear neutrophilic leucocytes, with dark blue nucleus, reddish lilac granules, and pale pink

cytoplasm; eosinophilic leucocytes, with blue nuclei, red to orange-red granules and blue cytoplasm; basophilic leucocytes, with purple or dark blue nucleus and granules dark purple (almost black); lymphocytes with nuclei dark purple and cytoplasm sky blue; platelets, with violet to purple granules.

Giemsa Stain

Identification: As in the case of Wright stain, no chemical methods for the identification or analysis of this stain have yet been devised. Like Wright stain it is a compound of eosin with methylene blue and its oxidation products.

Biological Tests: It is tested on dried thin films of blood which have been treated 5 to 7 minutes in methyl alcohol. The staining fluid is prepared according to the directions on the label, if such directions are furnished by the manufacturer; otherwise 0.5 g. of the powdered dye is dissolved in 33 ml. glycerin by standing at 55–60° C. for 1½ to 2 hours, then 33 ml. methyl alcohol is added and the solution allowed to stand 24 hours. The dried blood films are immersed in a staining fluid containing 30 drops of the above solution in 30 ml. distilled water. As different lots of stain vary in rapidity of action it is best to test a sample with three or four slides stained at varying periods from 15 to 40 minutes; it is not condemned if good results are obtained on any one of the slides. Results should be very similar to those secured with a good Wright stain, the chief difference being that the nuclei of the leucocytes are reddish purple instead of a dark violet.

It is also tested for staining unfixed thick blood films for the diagnosis of malaria. The films are made by spreading 3 to 5 drops of blood over a circle of about 15 mm. diameter on a scrupulously clean slide and air-drying for 18 to 24 hours at room temperature, kept in a horizontal position and protected from dust and insects. The smears are stained in diluted Giemsa stain, as above, or preferably diluted 1–50 in distilled water, buffered with phosphates to pH 7.0 or 7.2; they are then washed 5–10 minutes in distilled water or buffer solution of the above reaction and air dried. With a satisfactory sample, the malarial parasites should show with clear red chromatin and clear blue cytoplasm, while the red corpuscles do not show on account of the laking of the hemoglobin.

APPENDIX III

GENERAL LABORATORY INFORMATION

1. Solubility of Miscellaneous Compounds Used in Microscopic Work.*

	Per cent soluble in				
	Water				Alcohol
	20°C	25°C	30°C	100°C	
Ammonia alum	15.13	19.19	22.01	357.00	Insoluble
Copper sulfate ($5H_2O$)	42.31		48.81	203.3	Insoluble
Ferric chloride	74 or more				Quite soluble
Lithium carbonate	1.33			0.728	Insoluble
Mercuric chloride	7.39		8.43	53.9	49.5 at 25°
Potassium alum	11.40	14.14	16.58	422.00	
Potassium dichromate	12 to 13		18.13	102.00	Insoluble
Sodium sulfate (10 H_2O)	58.85	98.48	184.1	312.00	Insoluble
Urea	104.7		136.0		5.4 at 20°
Anilin	3.11 at 16°				
Tannic acid	20 to 300				50 to 400

2. Formulae of Fixing Solutions

1) Formol:
   Formalin (40% formaldehyde) ..... 4–10 parts
   Dist. water ..... 100 parts

2) Mercuric chloride:
   Sat. Aqu. (or Alc.) Sol. mercuric chloride
   5% Glacial acetic acid.

   The proportions of these two solutions to mix together must be determined experimentally in any instance, employing enough of the acetic acid to overcome the shrinking action of the sublimate.

3) Gilson's fluid.
   Nitric acid, 46° strength (about an 80% solution) ..... 15 cc.
   Glacial acetic acid ..... 4 cc.
   60% alcohol ..... 100 cc.
   Distilled water ..... 880 cc.
   Mercuric chloride ..... 20 g.

4) Sublimate acetic:
   Sat. Aqu. Sol. of mercuric chloride ..... 95 parts
   Glacial acetic acid ..... 5 parts

5) Mueller's fluid:
   Potassium bichromate ..... 25 g.
   Sodium sulfate ..... 10 g.
   Dist. water ..... 1000 cc.

*The data given in this table were obtained from various sources in the literature, and their accuracy is not vouched for; they are, however, sufficiently correct to guide anyone in making up a saturated solution of any of the above compounds.

6) Zenker's fluid:

Potassium bichromate	2.5 g.
Mercuric chloride	5–8 g.
Dist. water	ad 100 cc.
Glacial acetic acid	5 cc.

Dissolve the mercuric chloride and potassium bichromate in the water with the aid of heat. Do not add the acetic acid to the stock solution, but only to the part taken for hardening pieces of tissue.

7) Helly's fluid, or Zenker-formol:

This is a modification of Zenker in which the acetic acid is replaced by 5% formalin.

8) Carnoy's fluid:

Glacial acetic acid	1 part
Absolute alcohol	6 parts
Chloroform	3 parts

9) Formol-nitric:

Formol, 10%	3 parts
Nitric acid, 10%	1 part

10) Flemming's fluid. Strong formula.

(a)

Chromic acid, 1% Aqu. Sol	15 parts
Glacial acetic acid	1 part
Distilled water	4 parts

(b) Osmic acid, 2% in 1% chromic acid solution.

Just before using mix 4 parts of (a) with one part of (b). Use 10 times the volume of the object.

11) Bichromate-chromic-osmic acid mixture of Champy:

3% Aqu. Sol. of bichromate of potash	7 parts
1% chromic acid Aqu. Sol.	7 parts
Osmic acid, 2% Aqu. Sol.	4 parts

12) Platino-aceto-osmic acid mixture of Hermann:

Platinic chloride, 1% Aqu. Sol.	15 parts
Glacial acetic acid	1 part
Osmic acid, 2% Aqu. Sol.	2 parts

13) Merkel's fluid (F. E. V. Smith's modification):

Acetic acid, 5% Aqu. Sol.	100 cc.
Platinic chloride, 1% Aqu. Sol.	5 cc.
Chromic acid, 1% Aqu. Sol.	10 cc.

14) Bouin's fluid:

Sat. Aqu. Sol. picric acid	75 parts
Formol, C. P.	25 parts
Acetic acid, glacial	5 parts

15) Allen's fluid P. F. $A._3$:

Picric acid, Sat. Aqu. Sol.	75 parts
Formalin, C. P.	15 parts
Glacial acetic acid	10 parts
Urea	1 part

16) Farmer's:

Absolute alcohol	3 parts
Glacial acetic acid	1 part

17) Navashin's:
- (a) Chromic acid ..... 1.5 g.
  Glacial acetic acid ..... 10 cc.
  Distilled water ..... 90 cc.
- (b) Commercial formalin ..... 40 cc.
  Distilled water ..... 60 cc.

Mix equal parts (a) and (b) just before using.

18) Mann's:
- Picric acid ..... 1 g.
- Tannin ..... 2 g.
- Sat. $HgCl_2$–NaCl ..... 100 cc.

19) Van Gehuchtin's:
- Absolute alcohol ..... 60 cc.
- Chloroform ..... 30 cc.
- Glacial acetic acid ..... 10 cc.

3. Formula of Cleaning Fluid

For Slides, Cover Glasses, Etc.

Potassium or sodium bichromate (commercial grade satisfactory). 40 g.
Water ..... 150 cc.

Dissolve with a little heat if necessary, then cool to room temperature and add the following slowly:

Concentrated sulfuric acid ..... 230 cc.

APPENDIX IV

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1944c. Various oil soluble dyes as fat stains in the supersaturated isopropanol technic. *Stain Techn.*, **19**, *55–8.*
1944d. Various thiazin and fluorane dyes in paired combinations as neutral stains for tissues. *J. Tech. Methods*, **24**, *43–5.*
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1945b. Carycinel red and coccinel red, oil soluble anthraquinone dyes, as fat stains in animal histology. *Stain Techn.*, **20**, *73–5.*

1945c. Studies on selective staining of collagen with acid anilin dyes. *J. Techn. Methods*, **25**, *1–47*.
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1932. Romanowsky staining with buffered solutions. *Arch. Path.*, **14**, *515*.
1936. Id. II. Current modification. *J. Tech. Methods*, **14**, *65–70*.

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1935. Staining the skeleton of cleared embryos with alizarin red S. *Stain Techn.*, **10**, *61–3*.

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1940. Ersparnisse bei der Gonokokken- und Spirochätenfärbung. *Munch. Med. Woch.*, **87**, *888*. (Victoria blue 4R, either alone or with methyl green and pyronin, as a stain for Spirochaetes.)

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1885. Zur Färbetechnic. *Zts. Wis. Mikr.*, **2**, *145–56*. (Uses eosin preceding methyl green.)

LOOSANOFF, V. L.
1937. Use of Nile blue sulfate in marking starfish. *Science*, **85**, *2208*.

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1906. Methylene violet and methylene azur. *J. Inf. Dis.*, **3**, *412–33*. (The history of blood stains and the chemistry of its ingredients.)
1922. Tetrachrome blood stain; an economical and satisfactory imitation of Leishman's stain. *J. Am. Med. Assn.*, **78**, *1122*.
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1897. On certain improvements in histological technic. *J. Exp. Med.*, **2**, *529–33*. (Proposes formula for phosphotungstic acid hematoxylin.)
1900. A contribution to staining methods. *J. Exp. Med.*, **5**, *15–20*. (Anilin blue connective tissue stain described.)

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1938. Pathological Technic. Saunders, Philadelphia.

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1924. Pathological Technic. *Eighth Edition. Saunders, Philadelphia.*

Maneval, W. E.
1934. Rapid staining methods. *Science,* 80, *292–4.* Abs. in *Stain Techn.*, 10, *38.*
1941. Staining bacteria and yeasts with acid dyes. *Stain Techn.*, 16, *13–19*

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1948. Improvement in the method for the histochemical demonstration of alkaline phosphatase and its use in a study of normal and neoplastic tissue. *J. Nat. Cancer Inst.*, 9, *181–97.*

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1894. Ueber die Behandlung der Nervenzellen für experimentell-histologische Untersuchungen. *Zts. Wis. Mikr.*, 11, *479–94.* See p. 490. (Employs methyl blue with eosin.)
1902. Physiological Histology. *Clarendon Press, Oxford.*

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1933 a. Concerning fast green. *Stain Techn.*, 8, *73.*
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Margolena, L. A., and Hansen, P. A.
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1859. Pigmentlösung als Reagenz bei Mikroskopisch physiologisch Untersuchungen. *Bot. Zeitg.*, 17, *21–7. J. f. prakt Chem. v. Erdmann u. Wether,* 76, *37.* (First use of indigo.)

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1929. Some histological methods. Trichrome stainings and their preliminary technique. *J. Tech. Methods and Bul. Int. Assn. Med. Mus.*, 12, *75.*

Matsuura, S.
1925. Ueber die Färbung mit Kongorot. *Fol. Anat. Jap.*, 3, *107–10.* (Congo red for staining elastic tissue.)

Maurer, S., and Lewis, D.
1922. The cellular structure and differentiation of the specific cellular elements of the pars intermedia of the hypophysis of the domestic pig. *J. Exp. Med.*, 36, *141–56.* (In staining sections of the hypophysis, employs a mixture of acid violet and acid fuchsin, also a neutral stain combination of acid violet with safranin.)

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1878. Die Verwendbarkeit der Cochenille in der microscopischen Technic. *Zool. Anz.*, 1, *345–6.* (Uses cochineal with alum.)
1891. Ueber das Färben mit Hämatoxylin. *Mitt a. d. Zool. Stat. z. Neapel,* 10, *170–86.* (Hemalum, hemacalcium, etc.)
1892. Ueber das Färben mit Carmin, Cochenille und Hämatein Thonerde. *Mitt. a. d. Zool. Stat. z. Neapel,* 10, *480–504.*
1896. Ueber Schleimfärbung. *Mitt. a. d. Zool. Stat. z. Neapel,* 12, *303–30.* (Describes muci-carmine, muc-hematin and gluc-hematin.)
1899. Ueber Hämatoxylin, Carmin, und verwandte Materien. *Zts. Wis. Mikr.*, 16, *196–220.*

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1923. Haematoxylin. *Sci.*, 58, *515.*
1950. Handbook of Microscopical Technique. Third Edition, revised by Ruth McClung Jones. *Hoeber, New York.*

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1944. Picro- Mallory. *Stain Techn.*, **19**, *29–37*.

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1929. The finer histology of the normal glomerulus. *Amer. J. Path.*, **5**, *545–58*. (A modification of the Mallory-Heidenhain azocarmine-anilin-blue stain.)

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1944. Fluorescence microscopy applied to entomology and allied fields. *Stain Techn.*, **19**, *11–27*.

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1908. Die Spermatozytenteilungen bei der Hornisse. (*Vespa crabro* L.). *Arch. Mikr. Anat.*, **71**, *571–87*.

Michaelis, L.
1900. Die vitale Färbung, eine Darstellungsmethode der Zellgranula. *Arch. Mikr. Anat.*, **55**, *558*. (Uses Janus green for chondriosomes.)
1901. Ueber Fett Farbstoffe. *Virchow's Arch. f. Path. Anat. u. Phys.*, **164**, *263*. (Proposes Sudan IV.)

Mollier, G.
1938. Eine Vierfachfärbung zur Darstellung glatter und quergestreifter Muskulature und ihrer Beziehung zum Bindegewebe. *Zts. Wis. Mikr.*, **55**, *472–3*. (Use of orcein and naphthol green B in a quadruple stain.)

Monné, L.
1935. Permeability of the nuclear membrane to vital stains. *Proc. Soc. Exp. Biol. & Med.*, **32**, *1197–9*. (Use of azo blue.)
1938. Über experimentell hervorgerufene strukturelle Veränderungen der Golgiapparats und der Mitochondrien sowie über Bildung von Myelinfiguren in Spermatocyten und Spermatiden von *Helix lutescens*. *Protoplasma*, **30**, *582–91*.
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1933. The use of phenosafranin for staining fungi on culture media or in host tissue. *Science*, **77**, *23–4*. Abs. in *Stain Techn.*, **8**, *79*.

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1912. Kernstudien an Pflanzen. *Arch. f. Zellforschung.*, **8**, *Hft. 1, 1–51*. (Applies to plant pathology the stain mixture of Pianese—malachite green, acid fuchsin and martius yellow.)

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1919. Anwendungen der Abfangmethode auf die Bakteriengärungen. I. Acetaldehyd als Zwischenstufe bei der Vergärung von Zucker, Mannit und Glycerin durch *Bacterium coli*, durch Erreger der Ruhr und des Gasbrandes. *Biochem. Zts.*, **96**, *133–57*.

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1898. Zur Färbung der Malariaparasiten. *Centbl. f. Bakt. I Abt.*, **24**, *839–843*. (First to polychromize methylene blue intentionally in preparing blood stains.)

Ökland, F.
1939. Untersuchungen über Osteoblasten in Schliffen und Ausstrichen. *Zts. Wis. Mikr.*, **56**, *345*. (Nile blue sulfate for staining bone sections.)

Ono, K.
1934. Studien über die Färbung der Rekurrensspirochäten, insbesondere über den Zusammenhang der Färbung mit den Vorbehandlungen. *Acta dermatol. (Kyoto)*, **23**, *1–14, 39–55*. Abs. in *Stain Techn.*, **10**, *112*.

Orth, J.
1883. Notizen zur Färbetechnik. *Berl. Klin. Wochnsch.*, 20, *421–2*. (Proposes lithium carmine.)

Osborne, S. G.
1857. Vegetable cell structure and its formation as seen in the early stages of the growth of the wheat plant. *Trans. Micro. Soc.*, **5**, *104–22*. (Observes coloring of cell contents in plants grown in colored solutions—carmine, indigo, or vermilion.)

Paladino, Giovanni.
1895. Della nessuna partecipazione dell' epitelio della mucosa uterina e della relative glandole alla formazione della decidua vera e riflessa nella donna. *Rend. d. Accad. Sci. fisiche e matemat. Napoli*, **34**, *208–15*. (Biebrich scarlet with alum hematoxylin.)

Pappenheim, A.
1898. Befund von Smegmabacillen im menschlichen Lungenswurf. *Berl. Klin. Wochnsch.*, **37**, *809*. (Rosolic acid with methylene blue in the decolorizing solution following carbol-fuchsin in staining the tubercle organism.)

1899. Vergleichende Untersuchungen über die elementare Zuzammensetzung des rothen Knochenmarkes einige Säugethiere. *Virchow. Arch. f. Path. Anat. u. Phys.*, **157**, *19–76*. (Uses mixture of methyl green and pyronin.)

Parks, L. R., and Bartlett, P. G.
1935 a. Principles of dyeing with acid dyes. Amer. Dyestuff Rep., **24**, *476–8*.
1935 b. Principles of dyeing with basic dyes. Amer. Dyestuff Rep., **24**, *523–32*.

Pelagetti, M.
1904. Ueber einige neue Färbungsmethoden mit Anwendung der Zenkerschen Fixierungsflüssigkeit in der histologischen Technik der Haut. *Monatsch. f. Prak. Dermat.*, **38**, *532–6*. (Biebrich scarlet after

polychrome methylene blue or after Unna's hematein. Rose bengal following hematoxylin.)

PETER, KARL.
1899. Die Bedeutung der Nährzelle im Hoden. *Arch. Mikr. Anat.*, **53**, *180–211.* (Light green with hematoxylin.)

PETERSEN, HANS.
1924. Färben mit Säurealizarinblau. *Zts. Wis. Mikr.*, **41**, *363–5.*

PETERSON, A. R., CONN, H. J., and MELIN, C. G.
1933–4. Methods for the standardization of biological stains:
Part I. General considerations, *Stain Techn.*, **8**, *87.*
Part II. The fluorane derivatives, *Stain Techn.*, **8**, *95.*
Part III. Certain nitro and azo dyes, *Stain Techn.*, **8**, *121.*
Part IV. The triphenylmethane derivatives, *Stain Techn.*, **9**, *41.*
Part V. Miscellaneous dyes, *Stain Techn.*, **9**, *147.*

PETRAGNANI, G.
1928. Metodi rapidi di colorazione dei corpi del Negri. *Boll. Istit. Sierot. Milanese*, **7**, *557–61.* Abs. in *Stain Techn.*, **5**, *34.*

PETROFF, S. A.
1915. A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and feces. *J. Exper. Med.*, **21**, *38–42.* (Use of a gentian-violet-egg medium.)

PFITZER, E.
1883. Ueber ein Härtung und Färbung vereiniges Verfahren für die untersuchung des plasmatischen zelleibs. *Ber. Deut. Bot. Gesel.*, **1**, *44–7.* (Picro-nigrosin for chromatin.)

PHILLIPS, M., and COHEN, B.
1927. The preparation of vital neutral red. *Stain Techn.*, **2**, *74–9.*

PIANESE, G.
1896. Beitrag zur Histologie und Aetiologie des Carcinoms. *Beitrag zur Path. Anat. u. Allgem. Path., Suppl. I, 193 pp.* (Malachite green and martius yellow with acid fuchsin.)

PICK, J.
1935. Einige Vitalfärbungen am Frosch mit neuen fluoreszierenden Farbstoffen. *Zts. Wis. Mikr.*, **51**, 338–51.

PRATT, ROBERTSON, DUFRENOY, JEAN, and PICKERING, VIRGINIA L.
1948. Tetrazolium chloride, a valuable reagent in stain technology. *Stain Techn.*, **23**, *137–41.*

PRENANT, A.
1902. Contribution à l'étude de la ciliation. *Arch. d' Anat. Micr.*, **5**. (Light green with hematoxylin.)

PROESCHER, F.
1927. Oil red O pyridin, a rapid fat stain. *Stain Techn.*, **2**, *60–1.*
1933. Pinacyanol as a histological stain. *Proc. Soc. Exp. Biol. & Med.*, **31**, *79–81.* Abs. in *Stain Techn.*, **9**, *78.*
1934. Contribution to the staining of neuroglia. *Stain Techn.*, **9**, *33–8.*

PROESCHER F., and ARKUSH, A. S.
1928. Metallic lakes of the oxazines (gallimin blue, gallocyanin, and coelestin blue) as nuclear stain substitutes for hematoxylin. *Stain Techn.*, **3**, 28–38.

PRUDDEN, J. M.
1885. (A note without title). *Zts. Wis. Mikr.*, **2**, *288.* (In answer to a question by Flemming, gives formula of Delafield's hematoxylin.)

RAKIETEN, M. L., and RETTGER, L. F.
1927. Brilliant green and its use in an enrichment medium in the isolation of typhoid and paratyphoid organisms. *J. Inf. Dis.*, **41**, *93.*

Ramsdell, G. A., Johnson, W. T., and Evans, F. R.
1935. Investigation of resazurin as an indicator of the sanitary condition of milk. *J. Dairy Science*, **18**, *705–17*.

Ranvier.
1868. Technique microscopique. *Arch. de. Phys.* **1**, *No.* **2**, *319–21;* No. **5**, *666–70*. (First use of picro-carmine in a single procedure.)

Rawitz, B.
1899. Bemerkungen über Karminsäure und Hämatein. *Anat. Anz.*, **15**, *437–44*.

Reuter, Karl.
1901. Über den färbenden Bestandteil der Romanowsky-Nochtschen Malariaplasmodienfärbung, seine Reindarstellung und praktische Verwendung. *Centbl. f. Bakt. I Abt.*, **30**, *248–56*. (Dissolves precipitate of Nocht stain in absolute alcohol plus anilin oil.)

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1936. Alum cochineal-hematoxylin stain. Stain Tech., **11**, *166–7*.

Richards, O. W.
1943. Actinomyces of potato scab demonstrated by fluorescence microscopy. *Stain Techn.*, **18**, *91–4*.

Richards, O. W. and Miller, D. K.
1941. An efficient method for the identification of *M. tuberculosis* with a simple fluorescence microscope. *J. Clin. Path.*, **11**, *1–8*.

Robertson, O. H.
1917. The effects of experimental plethora on blood production. *J. Exp. Med.*, **26**, *221–37*. (Use of brilliant cresyl blue for staining reticulated blood cells.)

Robinson, H. C., and Rettger, L. F.
1916. Studies on the use of brilliant green and a modified Endo's medium in the isolation of *Bacillus typhosus* from feces. *J. Med. Res., N.S.*, **29**, *363–76*.

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1940. American azures in the preparation of satisfactory Giemsa stains for malaria parasites. Publ. Health Repts. **55**, 1272–8.

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1940. The inhibition of sulphate-reducing bacteria by dyestuffs. *J. Soc. Chem. Ind.*, **59**, *34–9*.

Romanovski, D. L.
1891. On the question of parasitology and therapy of malaria. (In Russian.) *Imp. Med. Military Acad., Dissert. No. 38, St. Petersburg, 1891*. (Proposes combination of eosin and methylene blue for staining blood.)
1891. Zur Frage der Parisitologie und Therapie der Malaria. *St. Petersb. Med. Wochenschr.*, **16**, *297–302, 307–15*. A slightly condensed version of the above.

Romeis, B.
1928. Taschenbuch der mikroskopischen Technik. *Twelfth Edition. Oldenbourg, Munich*.

Romell, L. G.
1934. A blue stain for microörganisms in humus and in soil. *Stain Techn.*, **9**, *141–5*.

Rothberger, C. J.
1898. Differential diagnostische Untersuchungen mit gefärbten Nährböden. *Centbl. f. Bakt. I Abt.*, **24**, *513–18*. (Neutral red as indicator in media for differentiating typhoid and colon bacilli.)

Ruth, E. B.
1946. Demonstration of the ground substance of cartilage, bone and teeth. *Stain Techn.*, **21**, *27–30*.

Sabin, Florence R.
1929. Chemical Agents: Supravital Stains. In *McClungs' Handbook of Microscopical Technique.* Hoeber, New York.

Sandiford, B. R.
1938. A new contrast stain for gonococci and meningococci in smears. *Brit. Med. J.*, **1**, *1155.*

Scales, F. M.
1922. A new method for differential staining of bacteria. *J. Inf. Dis.*, **31**, *494–8.*

Scanlan, J. T., French, R. W., and Holmes, W. C.
1927. Acid fuchsin as a stain—a refinement in manufacture. *Stain Techn.*, **2**, *50–5.*

Scanlan, J. T. and Melin, C. G.
1937. The production of basic fuchsin suitable for the Feulgen technic. *Stain Techn.*, **12**, *1–8.*

Schaffer.
1888. Die Färberei zum Studium der Knochenentwicklung. *Zts. Wis. Mikr.*, **5**, *1–19.* (Fast yellow for bone; Congo red for embryo sections.)

Schmorl, G.
1899. Darstellung feinere Knochenstructuren. *Centbl. Allge. Path. u. Path. Path. Anat.*, **10**, *745–9.*

Schoor, E.
1941. A new technic for staining vaginal smears: III. A single differential stain. *Science*, **94**, *545.*

Schultz, G.
1928–34. Farbstofftabellen. *7 Aufl. Leipzig. Bd. 1, 764 pp.; Bd. 2, 444 pp.; Erganzbd. 1, 182 pp.*

Schumacher, J.
1922. Welche chemische Substanz baut die Polkörnchen des Diphtheriebazillus auf? *Zentbl. Bakt., I Abt., Orig.* **88**, *362–6.* (Phosphine as a microchemical reagent.)

Schwartz, E.
1867. Ueber eine Methode doppelter Färbung Mikroskopischer Objecte und ihre Anwendung, etc. *Sitz. berichte d. k. Acad. d. Wiss: Wien Bd.* **55**, *Hft. 1, 671–89.* (First double staining.)

Schweiger-Seidel, F., and Dogiel, J.
1866. Ueber die peritoneal Hülle bei Frōschen und ihren zusammenhang mit dem Lymphgefässysteme. *Ber. d. k. Sachs. Gessel. d. Wiss. zu Leipzig*, **18**, *247–54.* (Use of carminates with acetic acid.)

Scott, R. E., and French, R. W.
1924 a. Standardization of biological stains. *Mil. Surg., Aug. 1924, 15 pp.*
1924 b. Standardization of biological stains. II. Methylen blue. *Mil. Surg., Sept. 1924, 16 pp.*
1924 c. Standardization of biological stains. III. Eosin and haematoxylin. *Mil. Surg., Nov. 1924, 8 pp.*

Scudder, Sara A.
1931. A differential stain favorable to the diagnosis of Neisserian infection. *Stain Techn.*, **6**, *99–105.*

Scudder, Sara A., and Lisa, J. R.
1931. A preliminary report on a combined Gram-Pappenheim stain for formalin fixed tissues. *Stain Techn.*, **6**, *51–2.*

Seligman, A. M.
1949. A new method for the histochemical demonstration of acid phosphatase. *J. Nat. Cancer Inst.*, **9**, *427–37.*

Shaeffer, Alice B., and Fulton, MacDonald.
1933. A simplified method of staining endospores. *Science*, **77**, *194*. Abs. in *Stain Techn.*, **8**, *80*.

Shaffer, R. W.
1933. A rapid method for distinguishing bleached sulfate from bleached sulfite. *Ind & Eng. Chem.*, **5**, *35*. (Brazilin for the microscopic differentiation of fibers.)

Shumway, Waldo.
1926. Fuchsin and picro-indigo-carmine, a polychromatic stain for vetebrate organogeny. *Stain Techn.*, **1**, *37–8*.

Singer, Marcus.
1952. Factors which control the staining of tissue sections with acid and basic dyes. *Internat. Rev. of Cytology*, **1952**, *211–55*.

Smith, F. E.
1951. Tetrazolium salt. *Science*, **113**, *751–4*.

Smith, G. M.
1915. The development of botanical microtechnique. *Trans. Am. Micro. Soc.*, **34**, *71–129*. (Includes discussion of early history of staining.)

Smith, H. A.
1939. A technic for making photomicrographic prints in color. *J. Tech. Methods*, **19**, *45–51*.

Smith, J. Lorrain.
1907. On the simultaneous staining of netural fat and fatty acid by oxazine dyes. *J. Path. & Bact.*, **12**, *1–4*. (Shows the possibility of differentiating fat and fatty acids in tissue by means of Nile blue sulfate.)

Smith, J. Lorrain, and Mair, W.
1911. Fats and lipoids in relation to methods of staining. *Skand. Arch. f. Physiol,*. **25**, *245–55*. (Describes the Nile blue sulfate technic for staining fat in sections, as well as other methods for fat staining.)

Smith, Louise.
1920. The hypobranchial apparatus of *Spelerpes bislineatus*. *J. Morph.*, **33**, *527–83*. (Use of methylene blue in staining cartilage by the Van Wijhe technic.)

Smith, N. R. and Dawson, Virginia T.
1944. The bacteriostatic action of rose bengal in media used for plate counts of soil fungi. *Soil Sci.*, **58**, *467–71*.

Society of Dyers and Colourists.
1924. Colour Index. Edited by F. M. Rowe. *Published by the Society, Bradford, Yorkshire, England.*

Soep, Leo.
1927. Le vert Janus en face du bleu de méthylène dans l'essai a la réductase selon Barthel. *Le Lait*, **7**, *927–35*. (Janus green as substitute for methylene blue in reductase test of milk.)

Spiridonovitch, R.
1924. Vital staining of white blood cells with cresylecht violet. *Anat. Rec.*, **27**, *367–73*.

Spuler, A.
1901. Ueber eine neue Stückfärbemethode. *Deut. Med. Wchnschr.*, **27**, *Vereine-Beilage No.* **14**, *116*. (Iron alum cochineal.)

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